PATENT

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APPLICATION FOR UNITED STATES LETTERS PATENT

for

METHODS AND COMPOSITIONS FOR VACCINATION COMPRISING NUCLEIC ACID AND/OR POLYPEPTIDE SEQUENCES OF *CHLAMYDIA*

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BACKGROUND OF THE INVENTION

The present application claims priority to co-pending U.S. Provisional Patent Application Serial No: 60/225,839 filed on December 15th, 2000. The entire text of the above-referenced disclosure is specifically incorporated herein by reference without disclaimer. The government owns rights in the present invention pursuant to DARPA grant number MDA 972-97-1-0013.

1. Field of the Invention

The present invention relates generally to the fields of immunology, bacteriology and molecular biology. More particularly, the invention relates to methods for screening and obtaining vaccines generated from the administration of expression libraries constructed from a *Chlamydia psittaci* genome or corresponding homologs from other *Chlamydia* species. In particular embodiments, it concerns methods and compositions for the vaccination of vertebrate animals against *Chlamydia* bacterial infections, wherein vaccination of the animal is *via* a protein or gene derived from part or all of the genes validated as vaccines.

2. Description of Related Art

Intracellular bacteria of the genus *Chlamydia* are important pathogens in both man and vertebrate animals, causing blindness in man, sexually transmitted disease, and community-acquired pneumonia, and most likely act as co-factors in atherosclerotic plaque formation in human coronary heart disease.

Ubiquitous *Chlamydia* (*C*) *psittaci* infections in cattle cause mastitis, infertility and abortion. A primary economic impact of *Chlamydia* in dairy cattle is the loss of milk production and quality. Serological evidence for infection with ruminant *Chlamydia psittaci* is found in virtually all cattle (Kaltenbock *et al.*, 1997). These infections typically do not cause overt signs of disease, but under stress of the host animal may elicit transient inflammation of the mammary gland and uterus. These stress-related herd health problems, while not clinically pronounced, result in major losses for animal agriculture due to reduced output and quality of animal products like milk.

Most existing vaccines for the treatment of bacterial infections are composed of live/attenuated or killed pathogens (Babiuk, 1999). Live/attenuated vaccines present the risk of residual, or reacquisition of, pathogenicity, and are associated with a high cost of production. In addition, efficacious live/attenuated vaccines cannot be developed against many pathogens, or are impractical to produce. Killed pathogens typically have less utility than live/attenuated vaccines, as they are not usually effective in eliciting cellular immune responses. An alternative is subunit vaccines that consist of one or a few proteins of the pathogen (Babiuk, 1999; Ellis, 1999). The proteins being developed for these vaccines are typically based on a dominant immune response in infected hosts, and/or on surmised importance in the disease process. Due to the high genetic complexity of bacteria or protozoa, the empirical approach to identify these proteins often requires extensive research on the pathogen's biology and produces a small, biased set of potential vaccine candidates. However, this is currently the only practical method when proteins are the commodity for testing a vaccine.

The development of genetic (DNA) immunization (Tang et al., 1992) not only offers a new method of vaccine delivery, but also enables a new, unbiased, approach to vaccine discovery. The inventors have proposed that the whole genome of a pathogen could be searched for protein vaccine candidates by directly assessing protection from challenge, termed expression library immunization (ELI) (U.S. Patent 5,703,057, specifically incorporated herein by reference). It involves making an expression library representing the whole genome of the pathogen in a genetic immunization vector. The library is subdivided into smaller groups, and DNA from each library is used to vaccinate animals that are subsequently challenged. The advantage of this approach is that all of the potentially protective genes could be discovered and used in any useful combination to reconstitute a vaccine devoid of non-protective, immunopathological, or immunosuppressive antigens. The potential of ELI was demonstrated in a murine *Mycoplasma pulmonis* infection, against which random *M. pulmonis* libraries were protective (Barry et al., 1995). Since then, others have reported on protective libraries (Brayton *et al.*, 1998; Piedrafita *et al.*, 1999), but the reduction of these libraries to individual genes has not been demonstrated.

As described above, the widespread human and animal infections by the genus *Chlamydia* represents a particular challenge for vaccinology. *Chlamydia psittaci* infections in cattle cause mastitis, infertility and abortion. A primary economic impact of *Chlamydia* in dairy cattle is the loss of milk production and quality. Thus, an effective vaccine against *Chlamydia* bacterial infections in cattle would be of great economic importance. However, there presently have been no effective vaccines developed against any *Chlamydia*.

SUMMARY OF THE INVENTION

The present invention overcomes these difficulties and problems in the art and provides for methods of immunization using *Chlamydia* antigens and polynucleotides. The instant invention relates to antigens and nucleic acids encoding such antigens obtainable by screening a *Chlamydia* genome. In more specific aspects, the invention relates to methods of isolating such antigens and nucleic acids and to methods of using such isolated antigens for producing immune responses. The ability of an antigen to produce an immune response may be employed in vaccination or antibody preparation techniques.

In some embodiments, the invention relates to isolated polynucleotides having a region that comprises a sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68 a complement of any of these sequences, or fragments thereof, or sequences closely related to these sequences. In some more specific embodiments, the invention relates to such polynucleotides comprising a region having a sequence comprising at least 17, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 125, 150, 200, or more contiguous nucleotides in common with at least one of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:24, SEQ ID

NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:58, SEQ ID NO:60, SEQ. ID NO:62, SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68 or its complement. Of course, such polynucleotides may comprise a region having all nucleotides in common with at least one of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68 or its complement.

In another aspect, the invention relates to polypeptides having sequences of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67, or SEQ ID NO:69 or fragments thereof, or sequences closely related to these sequences. The invention also relates to methods of producing such polypeptides using recombinant methods, for example, using the polynucleotides described above.

The invention relates to antibodies against *Chlamydia psittaci* antigens, including those directed against an antigen having sequences of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67, or SEQ ID NO:69 or an antigenic fragment thereof, or sequences closely related to these sequences. The antibodies may be polyclonal or monoclonal and produced by methods known in the art.

The invention contemplates vaccines comprising: (a) a pharmaceutically acceptable carrier, and (b) at least one polynucleotide having a *Chlamydia* sequence. In presently preferred embodiments, the at least one polynucleotide has a *Chlamydia psittaci, Chlamydia pneumoniae, Chlamydia trachomatis,* or *Chlamydia pecorum* sequence. In some specific embodiments at least one polynucleotide has a *Chlamydia psittaci* or *Chlamydia pneumonia* sequence.

The at least one polynucleotide may be isolated from a *Chlamydia* genomic DNA expression library but it need not be. For example, the polynucleotide may also be a sequence from one species that is determined to be protective based on the protective ability of a homologous sequence in another species. For example, the polynucleotide could be a *Chlamydia pneumonia* sequence that was determined to be protective after analysis of the *Chlamydia pneumonia* genomic sequence for homologues of *Chlamydia psittaci* antigens that had previously been shown to be protective. As discussed below, the polynucleotides need not be of natural origin, or to encode an antigen that is precisely a naturally occurring *Chlamydia* antigen. It is anticipated that polynucleotides and antigens within the scope of this invention my be synthetic and/or engineered to mimic, or improve upon, naturally occurring polynucleotides and still be useful in the invention.

In some embodiments, the at least one polynucleotide has a sequence isolated from *Chlamydia psittaci*, for example, a sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO52:, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO: 58, or SEQ ID NO:60, or fragment thereof, or sequences closely related to these sequences. In more specific such embodiments, the at least one polynucleotide has a sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, or SEQ ID NO:26, or fragment thereof, or sequences closely related to these sequences. In even more specific embodiments, the at least one polynucleotide has a sequence of SEQ ID NO:6, SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:20, or SEQ ID NO:24.

In some embodiments, the polynucleotide encodes an antigen having a sequence of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63; SEQ ID NO:65; SEQ ID NO:67; or SEQ ID NO:69; or antigenic fragment thereof, or sequences closely related to these sequences.

In many embodiments, the polynucleotide is comprised in a genetic immunization vector. Such a vector may, but need not, comprise a gene encoding a mouse ubiquitin fusion polypeptide. The vector, in some preferred embodiments, will comprise a promoter operable in eukaryotic cells, for example, but not limited to a CMV promoter. Such promoters are well known to those of skill in the art. In some embodiments, the polynucleotide is comprised in a viral expression vector, for example, but not limited to, a vector selected from the group consisting of adenovirus, adeno-associated virus, retrovirus and herpes-simplex virus.

The vaccines of the invention may comprise multiple polynucleotide sequences from one or more *Chlamydia* species. In some embodiments, the vaccine will comprise at least a first polynucleotide having a *Chlamydia psittaci* sequence and a second polynucleotide having a sequence, wherein the first polynucleotide and the second polynucleotide have different sequences. In some more specific embodiments, the first polynucleotide may have a sequence of SEQ ID NO:50.

The present invention also involves vaccines comprising: (a) a pharmaceutically acceptable carrier; and (b) at least one *Chlamydia* antigen. The at least one *Chlamydia* antigen can be a *Chlamydia psittaci, Chlamydia pneumoniae, Chlamydia trachomatis,* or *Chlamydia pecorum* antigen or an antigen of any other *Chlamydia* species. In some cases, the at least one *Chlamydia* antigen is a *Chlamydia psittaci* antigen. In some embodiments, the at least one *Chlamydia* antigen has a sequence of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID

NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63; SEQ ID NO:65; SEQ ID NO:67; or SEQ ID NO:69 or antigenic fragment thereof, or sequences closely related to these sequences. In some specific embodiments, the at least one *Chlamydia* antigen has a sequence of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, or SEQ ID NO:27, or an antigenic fragment thereof, or sequences closely related to these sequences. In even more specific embodiments, the at least one *Chlamydia* antigen has a sequence of SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:21, or SEQ ID NO:25.

The invention also relates to methods of immunizing an animal comprising providing to the animal at least one *Chlamydia* antigen, or antigenic fragment thereof, in an amount effective to induce an immune response. Again, the at least one *Chlamydia* antigen can be of *Chlamydia psittaci, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydia pecorum* or any other *Chlamydia* species. In some cases, the at least one *Chlamydia* antigen is a *Chlamydia psittaci* antigen, while in others it will not be. In further examples the *Chlamydia pneumoniae* antigens are comprised of SEQ ID NO: 63; EQ ID NO: 65; EQ ID NO: 675; EQ ID NO: 69: As discussed above, and described in detail below, the *Chlamydia* antigens useful in the invention need not be native antigens. Rather, these antigens may have sequences that have been modified in any number of ways known to those of skill in the art, so long as they result in or aid in an antigenic response.

In some embodiments of the invention, the provision of the at least one *Chlamydia* antigen comprises: (a) preparing a cloned expression library from fragmented genomic DNA, cDNA or sequenced genes of *Chlamydia*; (b) administering at least one clone of the library in a pharmaceutically acceptable carrier into the animal; and (c) expressing at least one *Chlamydia* antigen in the animal. The expression library may comprise at least one or more polynucleotides having a sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID

NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO52:, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO: 58, or SEQ ID NO:60, SEQ ID NO:62; SEQ ID NO:64; SEQ ID NO:66; or SEQ ID NO:68;or fragment thereof, or sequences closely related to these sequences. The expression library may be cloned in a genetic immunization vector, such as a vector of SEQ ID NO:1, or any other suitable vector. The vector may comprise a gene encoding a mouse ubiquitin fusion polypeptide designed to link the expression library polynucleotides to the ubiquitin gene. The vector may comprise a promoter operable in eukaryotic cells, for example a CMV promoter, or any other suitable promoter. In such methods, the polynucleotide may be administered by a intramuscular injection or epidermal injection. The polynucleotide may likewise be administered by intravenous, subcutaneous, intralesional, intraperitoneal, oral or inhaled routes of administration. In some specific, exemplary embodiments, the administration may be via intramuscular injection of at least 1.0 μg to 200 μg of the polynucleotide. In other exemplary embodiments, administration may be epidermal injection of at least 0.01 μg to 5.0 μg of the polynucleotide. In some cases, a second administration, for example, an intramuscular injection and/or epidermal injection, may administered at least about three weeks after the first administration. In these methods, the polynucleotide may be, but need not be, cloned into a viral expression vector, for example, a viral expression vector selected from the group consisting of adenovirus, herpes-simple virus, retrovirus and adeno-associated virus. The polynucleotide may also be administered in any other method disclosed herein or known to those of skill in the art.

In some embodiments, the provision of the *Chlamydia* antigen(s) may comprise: (a) preparing a pharmaceutical composition comprising at least one polynucleotide encoding a *Chlamydia* antigen or fragment thereof; (b) administering one or more clones of the library in a pharmaceutically acceptable carrier into the animal; and (c) expressing one or more *Chlamydia* antigens in the animal. The one or more polynucleotides can be comprised in one or more expression vectors, as described above and elsewhere in this specification.

Alternatively, the provision of the *Chlamydia* antigen(s) may comprise: (a) preparing a pharmaceutical composition of at least one *Chlamydia* antigen or an antigenic fragment thereof; and (b) administering the at least one antigen or fragment into the animal. The antigen(s) may be administered by a first intramuscular injection, intravenous injection, parenteral injection, epidermal injection, inhalation or oral route.

In preferred embodiments of the invention, the animal is a mammal. In some cases the mammal is a bovine, in others, the mammal is a human.

In some embodiments, these methods may induce an immune response against *Chlamydia psittaci.* Alternatively, these methods may be practiced in order to induce an immune response against a *Chlamydia* species other than *Chlamydia psittaci*, for example, but not limited to, *Chlamydia pneumoniae, Chlamydia trachomatis,* and/or *Chlamydia pecorum*. In some embodiments, these methods may be employed to induce an immune response against a non-*Chlamydia* infection or other disease.

These methods may comprise administering to the animal an antigen or antigenic fragment from a *Chlamydia* species other than *Chlamydia psittaci*. Also, these methods may comprise administering to the animal an antigen or antigenic fragment from a non-*Chlamydia* species.

This specification discusses methods of obtaining polynucleotide sequences effective for generating an immune response against the genus *Chlamydia* in a non-human animal comprising: (a) preparing a cloned expression library from fragmented genomic DNA of the genus *Chlamydia*; (b) administering one or more clones of the library in a pharmaceutically acceptable carrier into the animal in an amount effective to induce an immune response; and (c) selecting from the library the polynucleotide sequences that induce an immune response, wherein the immune response in the animal is protective against *Chlamydia* infection. Such methods may further comprise testing the animal for immune resistance against a *Chlamydia* bacterial infection by challenging the animal with *Chlamydia*. In some cases, the genomic DNA has been fragmented physically or by restriction enzymes, for example, but not limited to, fragments that average, about 200-1000 base pairs in length. In some cases, each clone in the library may comprise a gene encoding a mouse ubiquitin fusion polypeptide designed to link the expression library polynucleotides to the ubiquitin gene, but this is not required in all

cases. In some cases, the library may comprise about $1x10^3$ to about $1x10^6$ clones; in more specific cases, the library could have $1x10^5$ clones. In some preferred methods, about 0.01 μg to about 200 μg of DNA, from the clones is administered into the animal. In some situations the genomic DNA, cDNA or sequenced gene is introduced by intramuscular injection or epidermal injection. In some versions of these protocols, the cloned expression library further comprises a promoter operably linked to the DNA that permits expression in a vertebrate animal cell.

The application also discloses methods of preparing antigens that confer protection against infection in a vertebrate animal comprising the steps of: (a) preparing a cloned expression library from fragmented genomic DNA of the genus *Chlamydia*; (b) administering one or more clones of the library in a pharmaceutically acceptable carrier into the animal in an amount effective to induce an immune response; (c) selecting from the library the polynucleotide sequences that induce an immune response and expressing the polynucleotide sequences in cell culture; and (d) purifying the polypeptide(s) expressed in the cell culture. Often, these methods further comprise testing the animal for immune resistance against infection by challenging the animal with one or more *Chlamydia* or other pathogens.

The invention relates to methods of preparing antibodies against a *Chlamydia* antigen comprising the steps of: (a) identifying a *Chlamydia* antigen that confers immune resistance against *Chlamydia* bacterial infection when challenged with the *Chlamydia* species in which the antigen was prepared; (b) generating an immune response in a vertebrate animal with the antigen identified in step (a); and (c) obtaining antibodies produced in the animal.

The invention also relates to methods of assaying for the presence of *Chlamydia* infection in a vertebrate animal comprising: (a) obtaining an antibody directed against a *Chlamydia* antigen; (b) obtaining a sample from the animal; (c) admixing the antibody with the sample; and (d) assaying the sample for antigen-antibody binding, wherein the antigen-antibody binding indicates *Chlamydia* infection in the animal. In some cases, the antibody directed against the antigen is further defined as a polyclonal antibody. In others, the antibody directed against the antigen is further defined as a monoclonal antibody. In some embodiments, the *Chlamydia* antigen has a sequence of SEQ ID

NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63; SEQ ID NO:65; SEQ ID NO:67; or SEQ ID NO:69; or fragment thereof, or sequences closely related to these sequences. The assaying the sample for antigen-antibody binding may be by precipitation reaction, radioimmunoassay, ELISA, Western blot, immunofluorescence, or any other method known to those of skill in the art.

The invention also relates to kits for assaying a *Chlamydia* infection comprising, in a suitable container: (a) a pharmaceutically acceptable carrier; and (b) an antibody directed against a *Chlamydia* antigen.

The invention further relates to methods of assaying for the presence of a *Chlamydia* infection in an animal comprising: (a) obtaining an oligonucleotide probe comprising a sequence comprised within one of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO52:, SEQ ID NO:54, SEQ ID NO:56, SEQ ID NO: 58, or SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64; SEQ ID NO:66; or SEQ ID NO:68; or a complement thereof; and (b) employing the probe in a PCR or other detection protocol.

As used herein in the specification, "a" or "an" may mean one or more. As used herein, when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

As used herein, "plurality" means more than one. In certain specific aspects, a plurality may mean 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70,

71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 250, 300, 400, 500, 750, 1,000, 2,000, 3,000, 4,000, 5,000, 7,500, 10,000, 15,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000, 100,000, 125,000, 150,000, 200,000 or more, and any integer derivable therein, and any range derivable therein.

As used herein, "any integer derivable therein" means a integer between the numbers described in the specification, and "any range derivable therein" means any range selected from such numbers or integers.

As used herein, a "fragment" refers to a sequence having or having at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000 or more contiguous residues of the recited SEQ ID NOS, but less than the full-length of the SEQ. ID. NOS.. It is contemplated that the definition of "fragment" can be applied to amino acid and nucleic acid fragments.

As used herein, an "antigenic fragment" refers to a fragment, as defined above, that can elicit an immune response in an animal.

Reference to a sequence in an organism, such as a "*Chlamydia* sequence" refers to a segment of contiguous residues that is unique to that organism or that constitutes a fragment (or full-length region(s)) found in that organism (either amino acid or nucleic acid).

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better

understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Scheme for Expression Library Immunization.

FIG. 2. Production of the *Chlamydia psittaci* Library. The *Chlamydia psittaci* library was produced by first physically shearing the genomic DNA, strain BGM/B577, and size selecting fragments of 300-800 base pairs. The fragments were ligated into the *Bgl* II site of pCMVi-Ubs(+P3); see Sykes and Johnston, 1999 for details. The nucleotide sequence shown in this figure is given as SEQ ID NO:1.

FIG. 3. Flowchart depicting the process for deconvolution of the libraries. Each round consists of preparation of DNA samples, vaccination of mice, challenge and determination of the relative protection in each group.

FIG. 4. Results of protection assays in Rounds 1, 2 and 3. Protection was scored as lung weight relative to average of the vaccinated, maximum protection, positive control and the non-vaccinated, challenged, maximum disease, negative control. The relative protection score was calculated by assigning the score 1 to animals with lung weight equal to the vaccinated control and the score 0 to animals with lung weights equal to the challenged, non-vaccinated control. These points define a line; animals with lower lung weight, hence better protection, have a higher relative protection score. Animals that have worse disease than challenged, non-vaccinated controls, i.e. heavier lungs, will have a negative relative protection score. The unchallenged Naïve group consistently had lung weights slightly lower than the maximum protection, positive controls (Vaccinated) due to the peribronchiolar accumulation of lymphatic cells. In Rounds 2 and 3 the pools of plasmids from columns in the two-dimensional arrays are assigned numbers and the rows assigned letters. The solid bars indicate pools that were designated as protective and entered into the subsequent round. The error bars represent one standard deviation on either side of the mean.

FIG. 5. Results of protection assays of testing individual gene fragments in Round 4. Protection was scored as lung weight relative to the average of the vaccinated, maximum protection, positive control (Vaccinated=1) and the non-vaccinated, challenged, maximum disease, negative control (Challenged=0). The Pool<50AA is the DNA consisting of the pool of the 32 plasmids from Round 3 having predicted open-reading frames less than 50 amino acids long. Pool>50AA is the DNA consisting of all the 14 plasmids containing *Chlamydia psittaci* inserts encoding in-frame proteins more than 50 amino acids long. The numbers of each individual gene fragment tested correspond to the numbers in FIG. 4. The error bars represent one standard deviation of the mean.

FIG. 6. Summary of characterization of the single gene fragments of Round 4. The Relative Protection score of each *Chlamydia psittaci* (CP) gene fragment is provided along with the designation of the gene in *Chlamydia pneumonia* that has the highest similarity (*Chlamydia pneumonia* homologue). In two cases, gene fragment CP #4 and CP #12, the *Chlamydia psittaci* gene could also be identified. On the right is a linear map showing the location in each gene of the fragment that conferred protection (shaded).

FIG. 7. Protection data from DNA pools. CP1-6 is a negative pool from round 1. To test whether a single protective gene could be detected in a negative pool, 25 ng of either CP4 #4 or CP4 #11 was added to 50 μg of CP1-6.

FIG. 8. Protection against *Chlamydia pneumoniae* challenge by various homologs of *Chlamydia pneumoniae* from ELI-selected *Chlamydia psittaci* (CP) gene.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The widespread human and animal infections by the genus *Chlamydia* represents a particular challenge for vaccinology. For example, *Chlamydia psittaci* infections in cattle cause mastitis, infertility and abortion. A primary economic impact of *Chlamydia*

in dairy cattle is the loss of milk production and quality. Thus, an effective treatment for *Chlamydia* bacterial infections in human and other vertebrate animals would be of clinical and economic importance.

The present invention provides compositions and methods for the immunization of vertebrate animals, including humans, against infections using nucleic acid sequences and polypeptides elucidated by screening *Chlamydia psittaci*. These compositions and methods will be useful for immunization against *Chlamydia psittaci* bacterial infections and other infections and disease states. In particular embodiments, a vaccine composition directed against *Chlamydia* infections is provided. The vaccine according to the present invention comprises *Chlamydia* genes and polynucleotides identified by the inventors, that confer protective resistance in vertebrate animals to *Chlamydia* bacterial infections, and other infections. In other embodiments, the invention provides methods for immunizing an animal against *Chlamydia* infections, methods for preparing a cloned library *via* expression library immunization and methods for screening and identifying *Chlamydia* genes that confer protection against infection.

A. Expression Library Immunization

In particular embodiments, the immunization of vertebrate animals according to the present invention includes a cloned library of *Chlamydia* expression constructs. In specific embodiments, a cloned expression library of *Chlamydia psittaci* is provided. Expression library immunization, ELI herein, is well known in the art (U. S. Patent 5,703,057, specifically incorporated herein by reference). In certain embodiments, the invention provides an ELI method applicable to virtually any pathogen and requires no knowledge of the biological properties of the pathogen. The method operates on the assumption, generally accepted by those skilled in the art, that all the potential antigenic determinants of any pathogen are encoded in its genome. The inventors have previously devised methods of identifying vaccines using a genomic expression library representing all of the antigenic determinants of a pathogen (U. S. Patent 5,703,057). The method uses to its advantage the simplicity of genetic immunization to sort through a genome for immunological reagents in an unbiased, systematic fashion.

The preparation of an expression library is performed using the techniques and methods familiar one of skill in the art. The pathogen's genome, may or may not be known or possibly may even have been cloned. Thus one obtains DNA (or cDNA), representing substantially the entire genome of the pathogen (*e.g., Chlamydia psittaci)*. The DNA is broken up, by physical fragmentation or restriction endonuclease, into segments of some length so as to provide a library of about 10^5 (approximately 18x the genome size) members. The library is then tested by inoculating a subject with purified DNA of the library or sub-library and the subject challenged with a pathogen, wherein immune protection of the subject from pathogen challenge indicates a clone that confers a protective immune response against infection.

B. Nucleic Acids

The present invention provides *Chlamydia* polynucleotide compositions and methods that induce a protective immune response in vertebrate animals challenged with a *Chlamydia* bacterial infection. The preparation and purification of antigenic *Chlamydia* polypeptides, or fragments thereof (Section C) and antibody preparations directed against *Chlamydia* antigens, or fragments thereof (Section E) are described below.

Thus, in certain embodiments of the present invention, genes or polynucleotides encoding *Chlamydia* polypeptides or fragments thereof are provided. It is contemplated in other embodiments, that a polynucleotide encoding a *Chlamydia* polypeptide or polypeptide fragment will be expressed in prokaryotic or eukaryotic cells and the polypeptides purified for use as anti-*Chlamydia* antigens in the vaccination of vertebrate animals or in generating antibodies immunoreactive with *Chlamydia* polypeptides (*i.e.*, antigens). The genomes of *Chlamydia pneumoniae* and *Chlamydia trachomatis* have been completely sequenced. The *Chlamydia* genes are quite similar, with the four most protective genes identified being 30-71% identical and 45-85% similar in amino acid sequence.

Genes for various species of the genus *Chlamydia* have been cloned, identified and compared (Kalman *et al.*, 1999; Meijer *et al.*, 1999). For example, the genomes of *Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydia psittaci* and *Chlamydia pecorum* have been studied. The present invention is not limited in scope to the genes of

Chlamydia psittaci, however, as one of ordinary skill in the art could, using these nucleic acids, readily identify related homologues in various other species. In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a specific "*Chlamydia*" gene or polynucleotide fragment may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally indistinguishable, from the polynucleotide sequences disclosed herein by reference in U.S. Patent Application Serial No: 09/738,269 filed on December 15th , 2000.

1. Nucleic Acids Encoding *Chlamydia* Polypeptides

The present invention provides polynucleotides encoding antigenic *Chlamydia psittaci* polypeptides capable of inducing a protective immune response in vertebrate animals and for use as an antigen to generate anti-*Chlamydia psittaci* or other pathogen antibodies. In certain instances, it may be desirable to express *Chlamydia psittaci* polynucleotides encoding a particular antigenic *Chlamydia psittaci* polypeptide domain or sequence to be used as a vaccine or in generating anti-*Chlamydia psittaci* or other pathogen antibodies. Nucleic acids according to the present invention may encode an entire *Chlamydia psittaci* gene, or any other fragment of the *Chlamydia psittaci* sequences set forth herein. The nucleic acid may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the nucleic acid may comprise complementary DNA (cDNA). A protein may be derived from the designated sequences for use in a vaccine or to isolate useful antibodies.

The term "cDNA" is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic, non- or partially-processed RNA template, is that the cDNA primarily contains coding sequences of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, such as where the non-coding regions are required for optimal expression.

It also is contemplated that a given *Chlamydia* polynucleotide from a given species may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same polypeptide (see Table 2 below). In addition, it is

contemplated that a given *Chlamydia* polypeptide from a species may be generated using alternate codons that result in a different nucleic acid sequence but encodes the same polypeptide.

As used in this application, the term "a nucleic acid encoding a *Chlamydia* polynucleotide" refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine (Table 2, below), and also refers to codons that encode biologically equivalent amino acids, as discussed in the following pages.

TABLE 1

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG

Tyrosine	Tyr	Y	UAC UAU

Allowing for the degeneracy of the genetic code, sequences that have at least about 50%, usually at least about 60%, more usually about 70%, most usually about 80%, preferably at least about 90% and most preferably about 95% of nucleotides that are identical to the nucleotides of given *Chlamydia* gene or polynucleotide. Sequences that are essentially the same as those set forth in a *Chlamydia* gene or polynucleotide may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of a *Chlamydia* polynucleotide under standard conditions.

The DNA segments of the present invention include those encoding biologically functional equivalent *Chlamydia* proteins and peptides, as described above. Such sequences may arise as a consequence of codon redundancy and amino acid functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques or may be introduced randomly and screened later for the desired function, as described below.

2. Oligonucleotide Sequences

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary to the sequences of a *Chlamydia* polynucleotide. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of a *Chlamydia* polynucleotide under relatively stringent conditions such as those described herein. Such sequences may encode the entire *Chlamydia* polypeptide or functional or non-functional fragments thereof.

Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, 3000 or 3500 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions, or for vaccines..

Suitable hybridization conditions will be well known to those of skill in the art. In certain applications, for example, substitution of amino acids by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C. Formamide and SDS also may be used to alter the hybridization conditions.

One method of using probes and primers of the present invention is in the search for genes related to *Chlamydia* or, more particularly, homologues of *Chlamydia* from other species. Normally, the target DNA will be a genomic or cDNA library, although screening may involve analysis of RNA molecules. By varying the stringency of hybridization, and the region of the probe, different degrees of homology may be discovered.

Another way of exploiting probes and primers of the present invention is in site-directed, or site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to

complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

C. Polypeptides and Antigens

For the purposes of the present invention a *Chlamydia* polypeptide used as an antigen may be a naturally-occurring *Chlamydia* polypeptide that has been extracted using protein extraction techniques well known to those of skill in the art. In particular embodiments, a *Chlamydia* antigen is identified by ELI and prepared in a pharmaceutically acceptable carrier for the vaccination of an animal against *Chlamydia* infection.

In alternative embodiments, the *Chlamydia* polypeptide or antigen may be a synthetic peptide. In still other embodiments, the peptide may be a recombinant peptide produced through molecular engineering techniques. The present section describes the methods and compositions involved in producing a composition of *Chlamydia* polypeptides for use as antigens in the present invention.

1. *Chlamydia* Polypeptides as Antigens

Section A describes methods for preparing a cloned *Chlamydia* library *via* ELI. Described also are methods for screening and identifying *Chlamydia* genes that confer protection against *Chlamydia* infection. Thus, *Chlamydia* polypeptide encoding genes or their corresponding cDNA identified in the present invention can be inserted into an appropriate cloning vehicle for the production of *Chlamydia* polypeptides as antigens for the present invention. In addition, sequence variants of the polypeptide can be prepared.

These may, for instance, be minor sequence variants of the polypeptide that arise due to natural variation within the population or they may be homologues found in other species. They also may be sequences that do not occur naturally, but that are sufficiently similar that they function similarly and/or elicit an immune response that cross-reacts with natural forms of the polypeptide. Sequence variants can be prepared by standard methods of site-directed mutagenesis such as those described below in the following section.

Another synthetic or recombinant variation of a *Chlamydia*-antigen is a polyepitopic moiety comprising repeats of epitopic determinants found naturally on *Chlamydia* proteins. Such synthetic polyepitopic proteins can be made up of several homomeric repeats of any one *Chlamydia* protein epitope; or can comprise of two or more heteromeric epitopes expressed on one or several *Chlamydia* protein epitopes.

Amino acid sequence variants of the polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Insertional variants include fusion proteins such as those used to allow rapid purification of the polypeptide and also can include hybrid proteins containing sequences

from other proteins and polypeptides which are homologues of the polypeptide. For example, an insertional variant could include portions of the amino acid sequence of the polypeptide from one species, together with portions of the homologous polypeptide from another species. Other insertional variants can include those in which additional amino acids are introduced within the coding sequence of the polypeptide. These typically are smaller insertions than the fusion proteins described above and are introduced, for example, into a protease cleavage site.

In one embodiment, major antigenic determinants of the polypeptide may be identified by an empirical approach in which portions of the gene encoding the polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, the polymerase chain reaction (PCR) can be used to prepare a range of cDNAs encoding peptides lacking successively longer fragments of the C-terminus of the protein. The immunogenic activity of each of these peptides then identifies those fragments or domains of the polypeptide that are essential for this activity. Further experiments in which only a small number of amino acids are removed or added at each iteration then allows the location of other antigenic determinants of the polypeptide. Thus, the polymerase chain reaction, a technique for amplifying a specific segment of DNA *via* multiple cycles of denaturation-renaturation, using a thermostable DNA polymerase, deoxyribonucleotides and primer sequences is contemplated in the present invention (Mullis, 1990; Mullis *et al.*, 1992).

Another embodiment for the preparation of the polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. Because many proteins exert their biological activity *via* relatively small regions of their folded surfaces, their actions can be reproduced by much smaller designer (mimetic) molecules that retain the bioactive surfaces and have potentially improved pharmacokinetic/dynamic properties (Fairlie *et al.*, 1998).

The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. However, unlike proteins, peptides often lack well defined three dimensional structure in aqueous solution

and tend to be conformationally mobile. Progress has been made with the use of molecular constraints to stabilize the bioactive conformations. By affixing or incorporating templates that fix secondary and tertiary structures of small peptides, synthetic molecules (protein surface mimetics) can be devised to mimic the localized elements of protein structure that constitute bioactive surfaces. Methods for mimicking individual elements of secondary structure (helices, turns, strands, sheets) and for assembling their combinations into tertiary structures (helix bundles, multiple loops, helix-loop-helix motifs) have been reviewed (Fairlie *et al.*, 1998; Moore, 1994).

Methods for predicting, preparing, modifying, and screening mimetic peptides are described in U.S. Patent 5,933,819 and U.S. Patent 5,869,451 (each specifically incorporated herein by reference). It is contemplated in the present invention, that peptide mimetics will be useful in screening modulators of an immune response.

Modifications and changes may be made in the structure of a gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following data.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventor that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982).

It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of

the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

2. Synthetic Polypeptides

Contemplated in the present invention are Chlamydia Psittaci proteins and related peptides for use as antigens. In certain embodiments, the synthesis of a *Chlamydia* peptide fragment is considered. The peptides of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979), each incorporated herein by reference. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

3. Chlamydia Polypeptide/Antigen Purification

Chlamydia polypeptides, including *Chlamydia psittaci* polypeptides, of the present invention are used as antigens for inducing a protective immune response in an animal and for the preparation of anti-*Chlamydia* antibodies. Thus, certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of a *Chlamydia* polypeptide that is described herein above. The term "purified protein or peptide " as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will

generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain and adequate flow rate. Separation can be accomplished in a matter of minutes, or a most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by

covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

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D. Gene Delivery

In certain embodiments of the invention, an expression construct comprising a *Chlamydia* gene or other polynucleotide segment under the control of a heterologous promoter operable in eukaryotic cells is provided. For example, the delivery of 10 *Chlamydia psittaci*, antigen-encoding expression constructs can be provided in this manner. The general approach in certain aspects of the present invention is to provide a cell with an expression construct encoding a specific protein, polypeptide or peptide fragment, thereby permitting the antigenic expression of the protein, polypeptide or peptide fragment to take effect in the cell. Following delivery of the expression construct, the protein, 15 polypeptide or peptide fragment encoded by the expression construct is synthesized by the transcriptional and translational machinery of the cell, as well as any that may be provided by the expression construct.

Viral and non-viral vector systems are the two predominate categories for the delivery of an expression construct encoding a therapeutic protein, polypeptide, polypeptide 20 fragment. Both vector systems are described in the following sections. There also are two primary approaches utilized in the delivery of an expression construct for the purposes of gene therapy; either indirect, *ex vivo* methods or direct, *in vivo* methods. *Ex vivo* gene transfer comprises vector modification of (host) cells in culture and the administration or transplantation of the vector modified cells to a gene therapy recipient. *In vivo* gene transfer 25 comprises direct introduction of the vector (*e.g.*, injection, inhalation) into the target source or therapeutic gene recipient.

In certain embodiments of the invention, the nucleic acid encoding the gene or polynucleotide may be stably integrated into the genome of the cell. In yet further embodiments, the nucleic acid may be stably or transiently maintained in the cell as a 30 separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in

synchronization with the host cell cycle. How the expression construct is delivered to a cell and/or where in the cell the nucleic acid remains is dependent on the type of vector employed. The following gene delivery methods provide the framework for choosing and developing the most appropriate gene delivery system for a preferred application.

1. Non-Viral Polynucleotide Delivery

In one embodiment of the invention, a polynucleotide expression construct consists of naked recombinant DNA or plasmids. In preferred embodiments of the invention, an expression construct comprising, for example, a *Chlamydia psittaci* polynucleotide is administered to a subject via injection and/or particle bombardment (*e.g.*, a gene gun). Thus, in one preferred embodiment, polynucleotide expression constructs are transferred into cells by accelerating DNA-coated microprojectiles to a high velocity, allowing the DNA-coated microprojectiles to pierce cell membranes and enter cells. In another preferred embodiment, polynucleotides are administered to a subject by injection. Injection of a polynucleotide expression construct may be given by intramuscular, intravenous, subcutaneous, ir intraperitoneal injection, as long as the polynucleotide expression construct can effectively be delivered to a desired target.

a. Particle Bombardment

Particle Bombardment depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. The most commonly used forms rely on high-pressure helium gas (Sanford *et al.*, 1991), of which one of the present inventors is a co-inventor. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

For microprojectile bombardment transformation using the constructs of the instant invention, both physical and biological parameters may be optimized. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after

bombardment, such as the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, the orientation of an immature embryo or other target tissue relative to the particle trajectory, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids.

Accordingly, it is contemplated that one may wish to adjust various bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as DNA concentration, gap distance, flight distance, tissue distance, and helium pressure. It is further contemplated that the grade of helium may effect transformation efficiency. One also may optimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation.

Other physical factors include those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells immediately before and after bombardment. The pre-bombardment culturing conditions, such as osmotic environment, the bombardment parameters, and the plasmid configuration have been adjusted to yield the maximum numbers of stable transformants.

For microprojectile bombardment, one will attach (*i.e.*, "coat") DNA to the microprojectiles such that it is delivered to recipient cells in a form suitable for transformation thereof. In this respect, at least some of the transforming DNA must be available to the target cell for transformation to occur, while at the same time during delivery the DNA must be attached to the microprojectile. Therefore, availability of the transforming DNA from the microprojectile may comprise the physical reversal of interactions between transforming DNA and the microprojectile following delivery of the microprojectile to the target cell. This need not be the case, however, as availability to a target cell may occur as a result of breakage of unbound segments of DNA or of other molecules which comprise the physical attachment to the microprojectile. Availability may further occur as a result of breakage of bonds between the transforming DNA and other molecules, which are either directly or indirectly attached to the microprojectile. It

is further contemplated that transformation of a target cell may occur by way of direct illegitimate or homology-dependent recombination between the transforming DNA and the genomic DNA of the recipient cell. Therefore, as used herein, a "coated" microprojectile will be one which is capable of being used to transform a target cell, in that the transforming DNA will be delivered to the target cell, yet will be accessible to the target cell such that transformation may occur.

Any technique for coating microprojectiles which allows for delivery of transforming DNA to the target cells may be used. Methods for coating microprojectiles which have been demonstrated to work well with the current invention have been specifically disclosed herein. DNA may be bound to microprojectile particles using alternative techniques, however. For example, particles may be coated with streptavidin and DNA end labeled with long chain thiol cleavable biotinylated nucleotide chains. The DNA adheres to the particles due to the streptavidin-biotin interaction, but is released in the cell by reduction of the thiol linkage through reducing agents present in the cell.

Alternatively, particles may be prepared by functionalizing the surface of a gold oxide particle, providing free amine groups. DNA, having a strong negative charge, binds to the functionalized particles. Furthermore, charged particles may be deposited in controlled arrays on the surface of mylar flyer disks used in the PDS-1000 Biolistics device, thereby facilitating controlled distribution of particles delivered to target tissue.

b. Other Non-Viral Methods of Polynucleotide Delivery

Transfer of a cloned expression construct in the present invention also may be performed by any of the methods which physically or chemically permeabilize the cell membrane (e.g., calcium phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes and lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles and receptor-mediated transfection.

In certain embodiments, the use of lipid formulations and/or nanocapsules is contemplated for the introduction of *a Chlamydia psittaci* polynucleotide or polypeptide, or a gene therapy vector into host cells.

Nanocapsules can generally entrap compounds in a stable and/or reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and/or such particles may be easily made.

In a preferred embodiment of the invention, the polynucleotide or polypeptide may be associated with a lipid. The polynucleotide or polypeptide associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. The lipid or lipid/ polynucleotide or polypeptide associated compositions of the present invention are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape.

Lipids suitable for use according to the present invention can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma Chemical Co., dicetyl phosphate ("DCP") is obtained from K & K Laboratories (Plainview, NY); cholesterol ("Chol") is obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Preferably, chloroform is used as the only solvent since it is more readily evaporated than methanol.

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes may be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple

lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). However, the present invention also encompasses compositions that have different structures in solution than the normal vesicular structure. For example, the lipids may assume a micellar structure or merely exist as non-uniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Liposomes within the scope of the present invention can be prepared in accordance with known laboratory procedures, for example: the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis, as described in *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis ed. (1979) pp. 287-341, the contents of which are incorporated herein by reference; the method of Deamer and Uster (1983), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978).

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferring (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-

terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell type such as prostate, epithelial or tumor endothelial cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a *Chlamydia psittaci* gene or polynucleotide of interest may also be transferred in a similar manner *in vivo* and express the gene or polynucleotide product.

2. Viral Vectors

In certain embodiments, it is contemplated that a *Chlamydia psittaci* gene or other polynucleotide that confers immune resistance to infection pursuant to the invention may be delivered by a viral vector. The capacity of certain viral vectors to efficiently infect or enter cells, to integrate into a host cell genome and stably express viral genes, have led to the development and application of a number of different viral vector systems (Robbins *et al.*, 1998). Viral systems are currently being developed for use as vectors for *ex vivo* and *in vivo* gene transfer. For example, adenovirus, herpes-simple virus, retrovirus and adeno-associated virus vectors are being evaluated currently for treatment of diseases such as cancer, cystic fibrosis, Gaucher disease, renal disease and arthritis (Robbins and Ghivizzani, 1998; Imai *et al.*, 1998; U. S. Patent 5,670,488). The various viral vectors

described below, present specific advantages and disadvantages, depending on the particular gene-therapeutic application.

a. Adenoviral Vectors

In particular embodiments, an adenoviral expression vector is contemplated for the delivery of expression constructs. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue or cell-specific construct that has been cloned therein.

Adenoviruses comprise linear double stranded DNA, with a genome ranging from 30 to 35 kb in size (Reddy *et al.*, 1998; Morrison *et al.*, 1997; Chillon *et al.*, 1999). An adenovirus expression vector according to the present invention comprises a genetically engineered form of the adenovirus. Advantages of adenoviral gene transfer include the ability to infect a wide variety of cell types, including non-dividing cells, a mid-sized genome, ease of manipulation, high infectivity and they can be grown to high titers (Wilson, 1996). Further, adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner, without potential genotoxicity associated with other viral vectors. Adenoviruses also are structurally stable (Marienfeld *et al.*, 1999) and no genome rearrangement has been detected after extensive amplification (Parks *et al.*, 1997; Bett *et al.*, 1993).

Salient features of the adenovirus genome are an early region (E1, E2, E3 and E4 genes), an intermediate region (pIX gene, Iva2 gene), a late region (L1, L2, L3, L4 and L5 genes), a major late promoter (MLP), inverted-terminal-repeats (ITRs) and a ψ sequence (Zheng, *et al.*, 1999; Robbins *et al.*, 1998; Graham and Prevec, 1995). The early genes E1, E2, E3 and E4 are expressed from the virus after infection and encode polypeptides that regulate viral gene expression, cellular gene expression, viral replication, and inhibition of cellular apoptosis. Further on during viral infection, the MLP is activated, resulting in the expression of the late (L) genes, encoding polypeptides required for adenovirus encapsidation. The intermediate region encodes components of the adenoviral capsid. Adenoviral inverted terminal repeats (ITRs; 100-200 bp in length),

are *cis* elements, function as origins of replication and are necessary for viral DNA replication. The ψ sequence is required for the packaging of the adenoviral genome.

A common approach for generating an adenoviruses for use as a gene transfer vector is the deletion of the E1 gene (E1$^-$), which is involved in the induction of the E2, E3 and E4 promoters (Graham and Prevec, 1995). Subsequently, a therapeutic gene or genes can be inserted recombinantly in place of the E1 gene, wherein expression of the therapeutic gene(s) is driven by the E1 promoter or a heterologous promoter. The E1$^-$, replication-deficient virus is then proliferated in a "helper" cell line that provides the E1 polypeptides *in trans* (e.g., the human embryonic kidney cell line 293). Thus, in the present invention it may be convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. Alternatively, the E3 region, portions of the E4 region or both may be deleted, wherein a heterologous nucleic acid sequence under the control of a promoter operable in eukaryotic cells is inserted into the adenovirus genome for use in gene transfer (U. S. Patent 5,670,488; U. S. Patent 5,932,210, each specifically incorporated herein by reference).

Although adenovirus based vectors offer several unique advantages over other vector systems, they often are limited by vector immunogenicity, size constraints for insertion of recombinant genes and low levels of replication. The preparation of a recombinant adenovirus vector deleted of all open reading frames, comprising a full length dystrophin gene and the terminal repeats required for replication (Haecker *et al.*, 1997) offers some potentially promising advantages to the above mentioned adenoviral shortcomings. The vector was grown to high titer with a helper virus in 293 cells and was capable of efficiently transducing dystrophin in mdx mice, in myotubes *in vitro* and muscle fibers *in vivo*. Helper-dependent viral vectors are discussed below.

A major concern in using adenoviral vectors is the generation of a replication-competent virus during vector production in a packaging cell line or during gene therapy treatment of an individual. The generation of a replication-competent virus could pose serious threat of an unintended viral infection and pathological consequences for the patient. Armentano *et al.*, describe the preparation of a replication-defective adenovirus

vector, claimed to eliminate the potential for the inadvertent generation of a replication-competent adenovirus (U. S. Patent 5,824,544, specifically incorporated herein by reference). The replication-defective adenovirus method comprises a deleted E1 region and a relocated protein IX gene, wherein the vector expresses a heterologous, mammalian gene.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes and/or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo* (U. S. Patent 5,670,488; U. S. Patent 5,932,210; U. S. Patent 5,824,54). This group of viruses can be obtained in high titers, *e.g.*, 10^9 to 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. Many experiments, innovations, preclinical studies and clinical trials are currently under investigation for the use of adenoviruses as gene delivery vectors. For example, adenoviral gene delivery-based gene therapies are being developed for liver diseases (Han *et al.*, 1999), psychiatric diseases (Lesch, 1999), neurological diseases (Smith, 1998; Hermens and Verhaagen, 1998), coronary diseases (Feldman *et al.*, 1996), muscular diseases (Petrof, 1998), gastrointestinal diseases (Wu, 1998) and various cancers such as colorectal (Fujiwara and Tanaka, 1998; Dorai *et al.*, 1999), pancreatic (Carrion *et al.*, 1999), bladder (Irie *et al.*, 1999), head and neck (Blackwell *et al.*, 1999), breast (Stewart *et al.*, 1999), lung (Batra *et al.*, 1999) and ovarian (Vanderkwaak *et al.*, 1999).

b. Retroviral Vectors

In certain embodiments of the invention, the use of retroviruses for gene delivery are contemplated. Retroviruses are RNA viruses comprising an RNA genome. When a host cell is infected by a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. A particular advantage of retroviruses is that they can stably infect dividing cells with a gene of interest (*e.g.*, a therapeutic gene) by integrating into the host DNA, without expressing immunogenic viral proteins. Theoretically, the integrated retroviral vector will be maintained for the life of the infected host cell, expressing the gene of interest.

The retroviral genome and the proviral DNA have three genes: gag, pol, and env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication.

A recombinant retrovirus of the present invention may be genetically modified in such a way that some of the structural, infectious genes of the native virus have been removed and replaced instead with a nucleic acid sequence to be delivered to a target cell (U. S. Patent 5,858,744; U. S. Patent 5,739,018, each incorporated herein by reference). After infection of a cell by the virus, the virus injects its nucleic acid into the cell and the retrovirus genetic material can integrate into the host cell genome. The transferred retrovirus genetic material is then transcribed and translated into proteins within the host cell. As with other viral vector systems, the generation of a replication-competent retrovirus during vector production or during therapy is a major concern. Retroviral vectors suitable for use in the present invention are generally defective retroviral vectors that are capable of infecting the target cell, reverse transcribing their RNA genomes, and

integrating the reverse transcribed DNA into the target cell genome, but are incapable of replicating within the target cell to produce infectious retroviral particles (e.g., the retroviral genome transferred into the target cell is defective in gag, the gene encoding virion structural proteins, and/or in pol, the gene encoding reverse transcriptase). Thus, transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus.

The growth and maintenance of retroviruses is known in the art (U. S. Patent 5,955,331; U. S. Patent 5,888,502, each specifically incorporated herein by reference). Nolan *et al.* describe the production of stable high titre, helper-free retrovirus comprising a heterologous gene (U. S. Patent 5,830,725, specifically incorporated herein by reference). Methods for constructing packaging cell lines useful for the generation of helper-free recombinant retroviruses with amphoteric or ecotrophic host ranges, as well as methods of using the recombinant retroviruses to introduce a gene of interest into eukaryotic cells *in vivo* and *in vi*tro are contemplated in the present invention (U. S. Patent 5,955,331).

Currently, the majority of all clinical trials for vector mediated gene delivery use murine leukemia virus (MLV)-based retroviral vector gene delivery (Robbins *et al.*, 1998; Miller *et al.*, 1993). Disadvantages of retroviral gene delivery includes a requirement for ongoing cell division for stable infection and a coding capacity that prevents the delivery of large genes. However, recent development of vectors such as lentivirus (*e.g.*, HIV), simian immunodeficiency virus (SIV) and equine infectious-anemia virus (EIAV), which can infect certain non-dividing cells, potentially allow the *in vivo* use of retroviral vectors for gene therapy applications (Amado and Chen, 1999; Klimatcheva *et al.*, 1999; White *et al.*, 1999; Case et al., 1999). For example, HIV-based vectors have been used to infect non-dividing cells such as neurons (Takashi *et al.*, 1999; Miyake *et al.*, 1999), islets (Leibowitz *et al.*, 1999) and muscle cells (Johnston *et al.*, 1999). The therapeutic delivery of genes *via* retroviruses are currently being assessed for the treatment of various disorders such as inflammatory disease (Moldawer *et al.*, 1999), AIDS (Amado *et al.*, 1999; Engel and Kohn, 1999), cancer (Clay *et al.*, 1999), cerebrovascular disease (Weihl *et al.*, 1999) and hemophilia (Kay, 1998).

c. Herpes-Simplex Viral Vectors

Herpes simplex virus (HSV) type I and type II contain a double-stranded, linear DNA genome of approximately 150 kb, encoding 70-80 genes. Wild type HSV are able to infect cells lytically and to establish latency in certain cell types (*e.g.*, neurons). Similar to adenovirus, HSV also can infect a variety of cell types including muscle (Yeung *et al.*, 1999), ear (Derby *et al.*, 1999), eye (Kaufman *et al.*, 1999), tumors (Yoon *et al.*, 1999; Howard *et al.*, 1999), lung (Kohut *et al.*, 1998), neuronal (Garrido et al., 1999; Lachmann and Efstathiou, 1999), liver (Miytake *et al.*, 1999; Kooby et al., 1999) and pancreatic islets (Rabinovitch *et al.*, 1999).

HSV viral genes are transcribed by cellular RNA polymerase II and are temporally regulated, resulting in the transcription and subsequent synthesis of gene products in roughly three discernable phases or kinetic classes. These phases of genes are referred to as the Immediate Early (IE) or alpha genes, Early (E) or beta genes and Late (L) or gamma genes. Immediately following the arrival of the genome of a virus in the nucleus of a newly infected cell, the IE genes are transcribed. The efficient expression of these genes does not require prior viral protein synthesis. The products of IE genes are required to activate transcription and regulate the remainder of the viral genome.

For use in therapeutic gene delivery, HSV must be rendered replication-defective. Protocols for generating replication-defective HSV helper virus-free cell lines have been described (U. S. Patent 5,879,934; U. S. Patent 5,851,826, each specifically incorporated herein by reference in its entirety). One IE protein, Infected Cell Polypeptide 4 (ICP4), also known as alpha 4 or Vmw175, is absolutely required for both virus infectivity and the transition from IE to later transcription. Thus, due to its complex, multifunctional nature and central role in the regulation of HSV gene expression, ICP4 has typically been the target of HSV genetic studies.

Phenotypic studies of HSV viruses deleted of ICP4 indicate that such viruses will be potentially useful for gene transfer purposes (Krisky *et al.*, 1998a). One property of viruses deleted for ICP4 that makes them desirable for gene transfer is that they only express the five other IE genes: ICP0, ICP6, ICP27, ICP22 and ICP47 (DeLuca *et al.*,

1985), without the expression of viral genes encoding proteins that direct viral DNA synthesis, as well as the structural proteins of the virus. This property is desirable for minimizing possible deleterious effects on host cell metabolism or an immune response following gene transfer. Further deletion of IE genes ICP22 and ICP27, in addition to ICP4, substantially improve reduction of HSV cytotoxicity and prevented early and late viral gene expression (Krisky *et al.*, 1998b).

The therapeutic potential of HSV in gene transfer has been demonstrated in various *in vitro* model systems and *in vivo* for diseases such as Parkinson's (Yamada *et al.*, 1999), retinoblastoma (Hayashi *et al.*, 1999), intracerebral and intradermal tumors (Moriuchi *et al.*, 1998), B cell malignancies (Suzuki *et al.*, 1998), ovarian cancer (Wang *et al.*, 1998) and Duchenne muscular dystrophy (Huard *et al.*, 1997).

d. Adeno-associated Viral Vectors

Adeno-associated virus (AAV), a member of the parvovirus family, is a human virus that is increasingly being used for gene delivery therapeutics. AAV has several advantageous features not found in other viral systems. First, AAV can infect a wide range of host cells, including non-dividing cells. Second, AAV can infect cells from different species. Third, AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. For example, it is estimated that 80-85% of the human population has been exposed to AAV. Finally, AAV is stable at a wide range of physical and chemical conditions which lends itself to production, storage and transportation requirements.

The AAV genome is a linear, single-stranded DNA molecule containing 4681 nucleotides. The AAV genome generally comprises an internal non-repeating genome flanked on each end by inverted terminal repeats (ITRs) of approximately 145 bp in length. The ITRs have multiple functions, including origins of DNA replication, and as packaging signals for the viral genome. The internal non-repeated portion of the genome includes two large open reading frames, known as the AAV replication (rep) and capsid (cap) genes. The rep and cap genes code for viral proteins that allow the virus to replicate and package the viral genome into a virion. A family of at least four viral proteins are expressed from the AAV rep region, Rep 78, Rep 68, Rep 52, and Rep 40,

named according to their apparent molecular weight. The AAV cap region encodes at least three proteins, VP1, VP2, and VP3.

AAV is a helper-dependent virus requiring co-infection with a helper virus (*e.g.*, adenovirus, herpesvirus or vaccinia) in order to form AAV virions. In the absence of co-infection with a helper virus, AAV establishes a latent state in which the viral genome inserts into a host cell chromosome, but infectious virions are not produced. Subsequent infection by a helper virus "rescues" the integrated genome, allowing it to replicate and package its genome into infectious AAV virions. Although AAV can infect cells from different species, the helper virus must be of the same species as the host cell (*e.g.*, human AAV will replicate in canine cells co-infected with a canine adenovirus).

AAV has been engineered to deliver genes of interest by deleting the internal non-repeating portion of the AAV genome and inserting a heterologous gene between the ITRs. The heterologous gene may be functionally linked to a heterologous promoter (constitutive, cell-specific, or inducible) capable of driving gene expression in target cells. To produce infectious recombinant AAV (rAAV) containing a heterologous gene, a suitable producer cell line is transfected with a rAAV vector containing a heterologous gene. The producer cell is concurrently transfected with a second plasmid harboring the AAV rep and cap genes under the control of their respective endogenous promoters or heterologous promoters. Finally, the producer cell is infected with a helper virus.

Once these factors come together, the heterologous gene is replicated and packaged as though it were a wild-type AAV genome. When target cells are infected with the resulting rAAV virions, the heterologous gene enters and is expressed in the target cells. Because the target cells lack the rep and cap genes and the adenovirus helper genes, the rAAV cannot further replicate, package or form wild-type AAV.

The use of helper virus, however, presents a number of problems. First, the use of adenovirus in a rAAV production system causes the host cells to produce both rAAV and infectious adenovirus. The contaminating infectious adenovirus can be inactivated by heat treatment (56.degree. C. for 1 hour). Heat treatment, however, results in approximately a 50% drop in the titer of functional rAAV virions. Second, varying amounts of adenovirus proteins are present in these preparations. For example, approximately 50% or greater of the total protein obtained in such rAAV virion

preparations is free adenovirus fiber protein. If not completely removed, these adenovirus proteins have the potential of eliciting an immune response from the patient. Third, AAV vector production methods which employ a helper virus require the use and manipulation of large amounts of high titer infectious helper virus, which presents a number of health and safety concerns, particularly in regard to the use of a herpesvirus. Fourth, concomitant production of helper virus particles in rAAV virion producing cells diverts large amounts of host cellular resources away from rAAV virion production, potentially resulting in lower rAAV virion yields.

e. Other Viral Vectors

The development and utility of viral vectors for gene delivery is constantly improving and evolving. Other viral vectors such as poxvirus; *e.g.*, vaccinia virus (Gnant *et al.*, 1999; Gnant *et al.*, 1999), alpha virus; *e.g.*, sindbis virus, Semliki forest virus (Lundstrom, 1999), reovirus (Coffey *et al.*, 1998) and influenza A virus (Neumann *et al.*, 1999) are contemplated for use in the present invention and may be selected according to the requisite properties of the target system.

In certain embodiments, vaccinia viral vectors are contemplated for use in the present invention. Vaccinia virus is a particularly useful eukaryotic viral vector system for expressing heterologous genes. For example, when recombinant vaccinia virus is properly engineered, the proteins are synthesized, processed and transported to the plasma membrane. Vaccinia viruses as gene delivery vectors have recently been demonstrated to transfer genes to human tumor cells, *e.g.*, EMAP-II (Gnant *et al.*, 1999), inner ear (Derby *et al.*, 1999), glioma cells, *e.g.*, p53 (Timiryasova et al., 1999) and various mammalian cells, *e.g.*, P-450 (U. S. Patent 5,506,138). The preparation, growth and manipulation of vaccinia viruses are described in U. S. Patent 5,849,304 and U. S. Patent 5,506,138 (each specifically incorporated herein by reference).

In other embodiments, sindbis viral vectors are contemplated for use in gene delivery. Sindbis virus is a species of the alphavirus genus (Garoff and Li, 1998) which includes such important pathogens as Venezuelan, Western and Eastern equine encephalitis viruses (Sawai *et al.*, 1999; Mastrangelo *et al.*, 1999). *In vitro*, sindbis virus infects a variety of avian, mammalian, reptilian, and amphibian cells. The genome of

sindbis virus consists of a single molecule of single-stranded RNA, 11,703 nucleotides in length. The genomic RNA is infectious, is capped at the 5' terminus and polyadenylated at the 3' terminus, and serves as mRNA. Translation of a vaccinia virus 26S mRNA produces a polyprotein that is cleaved co- and post-translationally by a combination of viral and presumably host-encoded proteases to give the three virus structural proteins, a capsid protein (C) and the two envelope glycoproteins (El and PE2, precursors of the virion E2).

Three features of sindbis virus suggest that it would be a useful vector for the expression of heterologous genes. First, its wide host range, both in nature and in the laboratory. Second, gene expression occurs in the cytoplasm of the host cell and is rapid and efficient. Third, temperature-sensitive mutations in RNA synthesis are available that may be used to modulate the expression of heterologous coding sequences by simply shifting cultures to the non-permissive temperature at various time after infection. The growth and maintenance of sindbis virus is known in the art (U. S. Patent 5,217,879, specifically incorporated herein by reference).

f. Chimeric Viral Vectors

Chimeric or hybrid viral vectors are being developed for use in therapeutic gene delivery and are contemplated for use in the present invention. Chimeric poxviral/retroviral vectors (Holzer *et al.*, 1999), adenoviral/retroviral vectors (Feng *et al.*, 1997; Bilbao *et al.*, 1997; Caplen *et al.*, 1999) and adenoviral/adeno-associated viral vectors (Fisher *et al.*, 1996; U. S. Patent 5,871,982) have been described.

These "chimeric" viral gene transfer systems can exploit the favorable features of two or more parent viral species. For example, Wilson *et al.*, provide a chimeric vector construct which comprises a portion of an adenovirus, AAV 5' and 3' ITR sequences and a selected transgene, described below (U. S. Patent 5,871,983, specifically incorporate herein by reference).

The adenovirus/AAV chimeric virus uses adenovirus nucleic acid sequences as a shuttle to deliver a recombinant AAV/transgene genome to a target cell. The adenovirus nucleic acid sequences employed in the hybrid vector can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus

particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the hybrid viral production process by a selected packaging cell. At a minimum, the adenovirus nucleic acid sequences employed in the pAdA shuttle vector are adenovirus genomic sequences from which all viral genes are deleted and which contain only those adenovirus sequences required for packaging adenoviral genomic DNA into a preformed capsid head. More specifically, the adenovirus sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. The adenovirus sequences may be modified to contain desired deletions, substitutions, or mutations, provided that the desired function is not eliminated.

The AAV sequences useful in the above chimeric vector are the viral sequences from which the rep and cap polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences. These chimeras are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome (U. S. Patent 5,871,983, specifically incorporate herein by reference). In the hybrid vector construct, the AAV sequences are flanked by the selected adenovirus sequences discussed above. The 5' and 3' AAV ITR sequences themselves flank a selected transgene sequence and associated regulatory elements, described below. Thus, the sequence formed by the transgene and flanking 5' and 3' AAV sequences may be inserted at any deletion site in the adenovirus sequences of the vector. For example, the AAV sequences are desirably inserted at the site of the deleted E1a/E1b genes of the adenovirus. Alternatively, the AAV sequences may be inserted at an E3 deletion, E2a deletion, and so on. If only the adenovirus 5' ITR/packaging sequences and 3' ITR sequences are used in the hybrid virus, the AAV sequences are inserted between them.

The transgene sequence of the vector and recombinant virus can be a gene, a nucleic acid sequence or reverse transcript thereof, heterologous to the adenovirus sequence, which encodes a protein, polypeptide or peptide fragment of interest. The transgene is operatively linked to regulatory components in a manner which permits

transgene transcription. The composition of the transgene sequence will depend upon the use to which the resulting hybrid vector will be put. For example, one type of transgene sequence includes a therapeutic gene which expresses a desired gene product in a host cell. These therapeutic genes or nucleic acid sequences typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease.

E. *Chlamydia* Antibodies

In another aspect, the present invention provides antibody compositions that are immunoreactive with a *Chlamydia* polypeptide of the present invention, or any portion thereof.

An antibody can be a polyclonal or a monoclonal antibody. An antibody may also be monovalent or bivalent. A prototype antibody is an immunoglobulin composed by four polypeptide chains, two heavy and two light chains, held together by disulfide bonds. Each pair of heavy and light chains forms an antigen binding site, also defined as complementarity-determining region (CDR). Therefore, the prototype antibody has two CDRs, can bind two antigens, and because of this feature is defined bivalent. The prototype antibody can be split by a variety of biological or chemical means. Each half of the antibody can only bind one antigen and, therefore, is defined monovalent. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Howell and Lane, 1988).

Peptides corresponding to one or more antigenic determinants of a *Chlamydia* polypeptide of the present invention also can be prepared. Such peptides should generally be at least five or six amino acid residues in length, will preferably be about 10, 15, 20, 25 or about 30 amino acid residues in length, and may contain up to about 35-50 residues or so. Synthetic peptides will generally be about 35 residues long, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, CA). Longer peptides also may be prepared, *e.g.*, by recombinant means.

The identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity is taught in U.S. Patent 4,554,101 (Hopp), incorporated

herein by reference. Through the methods disclosed in Hopp, one of skill in the art would be able to identify epitopes from within an amino acid sequence such as a *Chlamydia* polypeptide sequence.

Numerous scientific publications have also been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou & Fasman, 1974a; Chou & Fasman, 1974b; Chou & Fasman, 1978a; Chou & Fasman, 1978b; Chou & Fasman, 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson & Wolf, 1988; Wolf *et al.*, 1988), the program PEPPLOT® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow & Bryant, 1993). Another commercially available software program capable of carrying out such analyses is MACVECTOR (IBI, New Haven, CT).

In further embodiments, major antigenic determinants of a *Chlamydia* polypeptide may be identified by an empirical approach in which portions of the gene encoding the polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, PCR can be used to prepare a range of peptides lacking successively longer fragments of the C-terminus of the protein. The immunoactivity of each of these peptides is determined to identify those fragments or domains of the polypeptide that are immunodominant. Further studies in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide to be more precisely determined.

Another method for determining the major antigenic determinants of a polypeptide is the SPOTS system (Genosys Biotechnologies, Inc., The Woodlands, TX). In this method, overlapping peptides are synthesized on a cellulose membrane, which following synthesis and deprotection, is screened using a polyclonal or monoclonal antibody. The antigenic determinants of the peptides which are initially identified can be further localized by performing subsequent syntheses of smaller peptides with larger

overlaps, and by eventually replacing individual amino acids at each position along the immunoreactive peptide.

Once one or more such analyses are completed, polypeptides are prepared that contain at least the essential features of one or more antigenic determinants. The peptides are then employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants also can be constructed and inserted into expression vectors by standard methods, for example, using PCR cloning methodology.

The use of such small peptides for antibody generation or vaccination typically requires conjugation of the peptide to an immunogenic carrier protein, such as hepatitis B surface antigen, keyhole limpet hemocyanin or bovine serum albumin. Methods for performing this conjugation are well known in the art.

1. Anti-*Chlamydia* Antibody Generation

The present invention provides monoclonal antibody compositions that are immunoreactive with a *Chlamydia* polypeptide. As detailed above, in addition to antibodies generated against a full length *Chlamydia* polypeptide, antibodies also may be generated in response to smaller constructs comprising epitopic core regions, including wild-type and mutant epitopes. In other embodiments of the invention, the use of anti-*Chlamydia* single chain antibodies, chimeric antibodies, diabodies and the like are contemplated.

As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

Monoclonal antibodies (mAbs) are recognized to have certain advantages, *e.g.*, reproducibility and large-scale production, and their use is generally preferred.

However, "humanized" *Chlamydia* antibodies also are contemplated, as are chimeric antibodies from mouse, rat, goat or other species, fusion proteins, single chain antibodies, diabodies, bispecific antibodies, and other engineered antibodies and fragments thereof. As defined herein, a "humanized" antibody comprises constant regions from a human antibody gene and variable regions from a non-human antibody

gene. A "chimeric antibody, comprises constant and variable regions from two genetically distinct individuals. An anti-*Chlamydia* humanized or chimeric antibody can be genetically engineered to comprise a *Chlamydia* antigen binding site of a given of molecular weight and biological lifetime, as long as the antibody retains its *Chlamydia* antigen binding site.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), chimeras and the like. Methods and techniques of producing the above antibody-based constructs and fragments are well known in the art (U.S. Patent 5,889,157; U.S. Patent 5,821,333; U.S. Patent 5,888,773, each specifically incorporated herein by reference).

U.S. Patent 5,889,157 describes a humanized B3 scFv antibody preparation. The B3 scFv is encoded from a recombinant, fused DNA molecule, that comprises a DNA sequence encoding humanized Fv heavy and light chain regions of a B3 antibody and a DNA sequence that encodes an effector molecule. The effector molecule can be any agent having a particular biological activity which is to be directed to a particular target cell or molecule. Described in U.S. Patent 5,888,773, is the preparation of scFv antibodies produced in eukaryotic cells, wherein the scFv antibodies are secreted from the eukaryotic cells into the cell culture medium and retain their biological activity. It is contemplated that similar methods for preparing multi-functional anti-*Chlamydia* fusion proteins, as described above, may be utilized in the present invention.

Means for preparing and characterizing antibodies also are well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference). The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic *Chlamydia* polypeptide composition in accordance with the present invention and collecting antisera from that immunized animal.

A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster,

a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin also can be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable molecule adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins or synthetic compositions.

Adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ-interferon, GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MHC antigens may even be used. Exemplary, often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

In addition to adjuvants, it may be desirable to coadminister biologic response modifiers (BRM), which have been shown to upregulate T cell immunity or downregulate suppressor cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (SmithKline Beecham, PA); low-dose Cyclophosphamide (CYP; 300 mg/m^2) (Johnson/ Mead, NJ), cytokines such as γ-interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B-7.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for

immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization.

A second, booster injection, also may be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

For production of rabbit polyclonal antibodies, the animal can be bled through an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or else the desired antibody fraction may be purified by well-known methods, such as affinity chromatography using another antibody, a peptide bound to a solid matrix, or by using, *e.g.*, protein A or protein G chromatography.

mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified *Chlamydia* polypeptide, peptide or domain, be it a wild-type or mutant composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells.

The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells also is possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

The animals are injected with antigen, generally as described above. The antigen may be coupled to carrier molecules such as keyhole limpet hemocyanin if necessary. The antigen would typically be mixed with adjuvant, such as Freund's complete or incomplete adjuvant. Booster injections with the same antigen would occur at

approximately two-week intervals, or the gene encoding the protein of interest can be directly injected.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible.

Often, a panel of animals will have been immunized and the spleen of an animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods also is appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. HAT medium, a growth medium containing hypoxanthine, aminopterin and thymidine, is well known in the art as a medium for selection of hybrid cells. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual

clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas then would be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. First, a sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion (*e.g.*, a syngeneic mouse). Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. Second, the individual cell lines could be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Fragments of the monoclonal antibodies of the invention can be obtained from the monoclonal antibodies so produced by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer.

It also is contemplated that a molecular cloning approach may be used to generate monoclonals. For this, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination which further increases the chance of finding appropriate antibodies.

Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in, for example, *E. coli*.

F. Pharmaceutical Compositions

Aqueous compositions of the present invention comprise an effective amount of a purified *Chlamydia* polynucleotide and/or a purified *Chlamydia* a protein, polypeptide, peptide, epitopic core region, and the like, dissolved and/or dispersed in a pharmaceutically acceptable carrier and/or aqueous medium. Aqueous compositions of gene therapy vectors expressing any of the foregoing are also contemplated.

The phrases "pharmaceutically and/or pharmacologically acceptable" refer to molecular entities and/or compositions that do not produce an adverse, allergic and/or other untoward reaction when administered to an animal.

As used herein, "pharmaceutically acceptable carrier" includes any and/or all solvents, dispersion media, coatings, antibacterial and/or antifungal agents, isotonic and/or absorption delaying agents and the like. The use of such media and/or agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media and/or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. For animal and more particularly human administration, preparations should meet sterility, pyrogenicity, general safety and/or purity standards as required by FDA Office of Biologics standards.

The biological material should be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. The active compounds may generally be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, intralesional, and/or even intraperitoneal routes, or formulated for oral or inhaled delivery. The preparation of an aqueous compositions that contain an effective amount of purified *Chlamydia* polynucleotide or polypeptide agent as an active component and/or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables,

either as liquid solutions and/or suspensions; solid forms suitable for using to prepare solutions and/or suspensions upon the addition of a liquid prior to injection can also be prepared; and/or the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions and/or dispersions; formulations including sesame oil, peanut oil and/or aqueous propylene glycol; and/or sterile powders for the extemporaneous preparation of sterile injectable solutions and/or dispersions. In all cases the form must be sterile and/or must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and/or storage and/or must be preserved against the contaminating action of microorganisms, such as bacteria and/or fungi.

Solutions of the active compounds as free base and/or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and/or in oils. Under ordinary conditions of storage and/or use, these preparations contain a preservative to prevent the growth of microorganisms.

A *Chlamydia* polynucleotide or polypeptide of the present invention can be formulated into a composition in a neutral and/or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and/or which are formed with inorganic acids such as, for example, hydrochloric and/or phosphoric acids, and/or such organic acids as acetic, oxalic, tartaric, mandelic, and/or the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, and/or ferric hydroxides, and/or such organic bases as isopropylamine, trimethylamine, histidine, procaine and/or the like. In terms of using peptide therapeutics as active ingredients, the technology of U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and/or 4,578,770, each incorporated herein by reference, may be used.

The carrier can also be a solvent and/or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and/or liquid polyethylene glycol, and/or the like), suitable mixtures thereof, and/or vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as

lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and/or antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and/or the like. In many cases, it will be preferable to include isotonic agents, for example, sugars and/or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and/or gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and/or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, and/or highly, concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and/or in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and/or the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and/or the liquid diluent first rendered isotonic with sufficient saline and/or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and/or intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be

known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and/or either added to 1000 ml of hypodermoclysis fluid and/or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and/or 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

A *Chlamydia* polynucleotide or protein-derived peptides and/or agents may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, and/or about 0.001 to 0.1 milligrams, and/or about 0.1 to 1.0 and/or even about 10 milligrams per dose and/or so. Multiple doses can also be administered.

In addition to the compounds formulated for parenteral administration, such as intravenous and/or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets and/or other solids for oral administration; liposomal formulations; time release capsules; and/or any other form currently used, including cremes.

One may also use nasal solutions and/or sprays, aerosols and/or inhalants in the present invention. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops and/or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and/or slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and/or appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and/or include, for example, antibiotics and/or antihistamines and/or are used for asthma prophylaxis.

Additional formulations which are suitable for other modes of administration include vaginal suppositories and/or pessaries. A rectal pessary and/or suppository may also be used. Suppositories are solid dosage forms of various weights and/or shapes, usually medicated, for insertion into the rectum, vagina and/or the urethra. After insertion, suppositories soften, melt and/or dissolve in the cavity fluids. In general, for suppositories, traditional binders and/or carriers may include, for example, polyalkylene glycols and/or

triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and/or the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations and/or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent and/or assimilable edible carrier, and/or they may be enclosed in hard and/or soft shell gelatin capsule, and/or they may be compressed into tablets, and/or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and/or used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and/or the like. Such compositions and/or preparations should contain at least 0.1% of active compound. The percentage of the compositions and/or preparations may, of course, be varied and/or may conveniently be between about 2 to about 75% of the weight of the unit, and/or preferably between 25-60%. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and/or the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, and/or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and/or the like; a lubricant, such as magnesium stearate; and/or a sweetening agent, such as sucrose, lactose and/or saccharin may be added and/or a flavoring agent, such as peppermint, oil of wintergreen, and/or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings and/or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, and/or capsules may be coated with shellac, sugar and/or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and/or propylparabens as preservatives, a dye and/or flavoring, such as cherry and/or orange flavor.

G. Kits

Therapeutic kits of the present invention are kits comprising a *Chlamydia* polynucleotide or polypeptide or an antibody to the polypeptide. Such kits will generally contain, in a suitable container, a pharmaceutically acceptable formulation of a *Chlamydia* polynucleotide or polypeptide, or an antibody to the polypeptide, or vector expressing any of the foregoing in a pharmaceutically acceptable formulation. The kit may have a single container, and/or it may have a distinct container for each compound.

When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The *Chlamydia* polynucleotide or polypeptide, or antibody compositions may also be formulated into a syringeable composition. In which case, the container may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit.

However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container.

The container will generally include at least one vial, test tube, flask, bottle, syringe and/or other container, into which the *Chlamydia* polynucleotide or polypeptide, or antibody formulation are placed, preferably, suitably allocated. The kits may also comprise a second container for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, *e.g.*, injection and/or blow-molded plastic containers into which the desired vials are retained.

Irrespective of the number and/or type of containers, the kits of the invention may also comprise, and/or be packaged with, an instrument for assisting with the injection/administration and/or placement of the ultimate *Chlamydia* polynucleotide or polypeptide,or an antibody to the polypeptide within the body of an animal. Such an

instrument may be a syringe, pipette, forceps, and/or any such medically approved delivery vehicle.

H. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Exemplary ELI Protocol

The following sections outline general methodology that one might use to prepare, screen and utilize ELI according to the present invention. Of course the following methods are merely general guidelines and should not limit one of skill in the art from modifying the present invention to accomplish a desired goal using ELI.

1. Library Construction

The present invention provides expression library constructs of genus *Chlamydia psittacii*. An expression library of *Chlamydia psittaci* can be produced by first physically shearing the genomic DNA of *Chlamydia psittaci* (*e.g.*, *Chlamydia psittaci* strain B577) and size-selecting fragments of 300-800 base pairs. The protocol used by the present inventors to produce a *Chlamydia psittaci* library is similar to that described in Sykes and Johnston (1999). Adaptors were added and the DNA fragments ligated into a genetic immunization vector (FIG. 2) designed to link fragments to the mouse ubiquitin gene. However, the fragments can be blunt-end cloned.

This vector is known to enhance MHC class I-restricted immune responses (Sykes and Johnston, 1999), while sterilizing immunity against *Chlamydia* is thought to be MHC

class II-dependent (Morrison *et al.*, 1995). However, any genetic immunization procedure, by the mechanism of intracellular expression of the inserted genes, will target towards class I antigen presentation. Nevertheless, both MHC class I- and class II-restricted immune responses to the expressed antigens are well documented (Barry *et al.*, 1995; Sykes and Johnston, 1999). The inventors observed, for instance, pronounced delayed-type hypersensitivity responses, mediated by MHC II-restricted $CD4^+$ Th1 cells, against protective *Chlamydia psittaci* B577 antigens, which were expressed from the ubiquitin fusion vector. In addition to the fact that MHC II-restricted immunity is generated by the ubiquitin fusion vector, MHC I-restricted immunity appears to mediate protection in the early phase of chlamydial infection (Morrison et al., 1995; Rottenberg et al., 1999). This duality of the cellular immune response generated by the ubiquitin fusion vector might explain the efficacy of this vector for genetic immunization against intracellular bacteria.

A library of approximately 82,000 individual members was created and tested as 27 sub-libraries each with 2,400-3,400 plasmid clones. The average insert frequency was approximately 67% and the average insert size was 660 base pairs. Nitrocellulose replica filters were made of each original colony plating of a sub-library pool for subsequent retrieval of positive clones. This generated a library with approximately six-fold expression-equivalent redundancy. One expression equivalent is defined as the number of in-frame fragments necessary to completely represent all authentic open reading frames. Since the genome size of *Chlamydia psittaci* is approximately $1x10^6$ base pairs and only one-sixth of the actual open-reading frames will be cloned in the right orientation and frame, it requires at least six genomic equivalents to encode one expression equivalent. Each sub-library was propagated on plates and harvested to prepare DNA. DNA representing each sub-library was used for genetic immunization of mice in the following section.

2. Vaccination and Challenge

For the first round of testing, outbred, 6-week old, female NIH-Swiss Webster mice were inoculated with the purified DNA of each sub-library using both intra-muscular (i.m.) and epidermal injection. The epidermal injection was effected with a

gene gun (Sanford et al., 1991). Each mouse was given 50 μg DNA i.m. and 5 μg DNA by gene gun. It has been argued that the gene gun immunization favors a Th2 and the i.m. injection a Th1 type response (Feltquate et al., 1997), therefore both types of injection were given to each group. In the first round of testing, the prime inoculation was followed by a boost 9 weeks later, before intranasal challenge with 3 x 10^6 inclusion forming units (IFU) of *Chlamydia psittaci* strain B577 13 weeks after prime inoculation. The animals were sacrificed 12 days after the challenge, and lungs were weighed.

3. Library Deconvolution

The basic scheme for handling the reduction of the libraries is depicted in FIG. 3. Fourteen groups out of the first round looked promising, so the individual clones from these groups were picked and grown in 96 well microtiter plates. This gave approximately 40,000 wells in microtiter plates, therefore about 40,000 clones. The second round was reduced using a two dimensional array format. As depicted in FIG. 3, the DNA was prepared from colonies pooled from rows and columns of the array. The rationale was that if a row and column conferred protection, the colonies at the intersection would be responsible. This scheme is premised on largely additive effects of the protective clones. This 24x24 array yielded pools of ~1,700 clones with each intercession having ~96 clones. Currently the inventors deconvolute the second round with a 3-dimesional array.

Since the lung weight was highly variable in the outbred NIH-Swiss mice with variable MHC background, the inventors decided to use inbred BALB/c mice in subsequent rounds. The 48 DNA pools for round two were i.m. injected into BALB/c mice at 50 μg DNA/animal, and the animals were boosted at seven weeks by both gene gun inoculation and i.m. injection. The mice were given a higher *Chlamydia psittaci* challenge, 1.6 x 10^6 IFU *Chlamydia psittaci* B577, at approximately 12 weeks, again to further differentiate the groups. Animals were sacrificed and results evaluated as in round one.

In the fourth round, the animals received two boosts rather than one, and the challenge inoculum was increased to 3 x10^6 IFU *Chlamydia psittaci* B577 to increase the selectivity of protection scoring. Furthermore, because too much DNA may lead to a

decrease in cellular immune response, the amount of each individual clone was reduced by half, with the difference made up with pUC118 DNA, so each mouse received a total 50 μg DNA for i.m. immunization, but only 25 μg of the specific clone. The inventors also decreased the gene gun DNA in the same manner: 1.25 μg/ear of the specific clone and 1.25 μg pUC118. Mice were boosted i.m. at both four and nine weeks after prime inoculation, and were challenged. The results of this final round are depicted in FIG. 5.

4. Analysis of Sequences

The clones conferring protection were re-sequenced and then compared by BLAST search to Genbank and particularly to the recently completed *Chlamydia pneumoniae* (Kalman et al., 1999) genome sequences (FIG. 6). Of the 14 single genes identified in this study, ten are internal fragments and three contain the C-terminus of the protein. Of the five most protective clones, one was from a putative outer membrane protein and one was from a cell surface protein. The other three were from cytosolic proteins.

Four of the 14 clones have sequence similarity to a class of proteins known as putative outer membrane proteins (POMPs) in *Chlamydia psittaci* and *Chlamydia pneumoniae*. Many of the "putative" outer membrane proteins are known to be localized to the outer membrane and to be highly immunogenic (Longbottom et al., 1996; Tan et al., 1990).

5. Mixing Experiment

The two dimensional approach used to find protective gene fragments assumes that the protection is due to a single highly protective gene within a pool. To verify that such genes would be found, 25 ng (i.e. 1/2000) of either of the two most protective genes was added to a pool that scored negative (pool 6 round 1). As depicted in FIG. 7, spiking with either clone converted the negative library to a positive.

Example 2

Materials and Methods

Library construction. *Chlamydia psittaci* strain B577 (ATCC VR-656) was grown in BGMK cells and elementary bodies (EB) were purified by renograff gradient centrifugation as described (Huang et al., 1999). Genomic DNA was isolated from EB by proteinase K and RNase digestion followed by cetyl-trimethyl ammonium bromide (Kaltenbock et al., 1997).

Genomic DNA was physically sheared using a nebulizer (Glas Col, Terra Haute, IN), then size fractioned on a 1.5% TBE agarose gel. Agarose with fragments between 300-700 base pairs was excised and the DNA was electroeluted. Adaptors (top strand 5': GATCTGGATCCCGAT (SEQ ID NO.2) ATCGGGCTCCA (SEQ ID NO.3) onto the fragments, then the fragments were cloned into pCMVi-UBs at the Bgl II site (See FIG. 6 and Sykes and Johnston, 1999 for more details). The ligations were transformed into DH 5 alpha electrocompetent cells and plated onto 150 mm diameter YT-Ampicillin (75 µg/mL final concentration) plates. The resulting plates had between 2400-3400 individual clones per plate. After the plates were incubated overnight at 37°C, the colonies from were lifted using nitrocellulose filters soaked in L-Broth with 8% DMSO, and these filters were stored at –80 °C. The original agar plates were then incubated at 37 °C for an additional six hours. Ten mL of L Broth was added to each plate, the E. coli was scraped into 150 mL of L-Broth and grown at 37 °C for 30 minutes. Ampicillin was then added to a final concentration of 50 µg/mL, and the cultures were grown overnight at 37 °C. Cells were pelleted and the DNA was purified using Qiagen tip 500 columns.

Inoculation of DNA. Round One: DNA from the pools was injected into 6-week old female NIH-Swiss mice. All mice received 50 µg total DNA by i.m. injections, evenly distributed between the quadriceps and tibialis anterior muscles. Eighteen of the groups also received gene gun inoculations (wand), with 2.5 µg DNA inoculated into each ear. The animals were boosted once at nine weeks in the same manner as the primary inoculation – all mice received i.m. injections, but only the same 18 groups received gene gun injections -- then intranasally challenged with 5.5 x 10^5 IFU of *Chlamydia psittaci* strain B577 at 13 weeks. The mice were sacrificed 11 days after the challenge, and lungs were weighed.

Round Two: Nitrocellulose filters from the positive pools were placed on L-Broth Bio-Assay plates supplemented with 75 μg/mL ampicillin and 2% agar. The filters were incubated on the plates for approximately 15 minutes, then the nitrocellulose was discarded. The colonies were grown at 30°C for 12 hours. The majority of the colonies were picked into 96 well microtiter plates containing HYT media (1.6% Bacto-tryptone, 1.0% Bacto-yeast extract, 85.5 mM NaCl, 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM Sodium citrate, 0.4 mM MgSO4, 6.8 mM ammonium sulfate, 4.4 % wt/vol glycerol) supplemented with 75 μg/mL ampicillin, using a Hybaid colony picker; the plates were then visually inspected and the remainder of the colonies were hand-picked. The microtiter plates were designated by their original pool number and by the order in which they were picked. Hence, plate 5.10 was from original pool 5 and was the tenth plate picked. The colonies were subdivided into groups as is indicated in FIG. 2. All of the microtiter plates comprising a pool were stamped onto on L-Broth Bio-Assay plates supplemented with 75 μg/mL ampicillin and were grown overnight at 37°C. The cells from these plates were harvested by adding L-Broth to the plates and scraping off the cells. The cells were pelleted by centrifugation then resuspended in Qiagen buffer P1. The remainder of the DNA prep proceeded according to manufacture's instructions.

These 48 DNA pools were i.m. injected into 6-week old BALB/c mice at 50 μg DNA/animal. For the initial inoculation, the mice did not receive gene gun inoculations. At seven weeks, the mice were boosted with 50 μg DNA/animal. In addition to the i.m. injections, the first 31 groups received gene gun (Rumsey-Loomis) inoculations at 2.5 μg DNA/ear; however, the gene gun failed at group 32, and the last 17 groups received only i.m. injections. The mice were given a higher challenge, 1.6×10^6 IFU *Chlamydia psittaci* B577, at 12 weeks. Animals were sacrificed as in round one.

Round Three: Colonies from the microtiter plates that were judged to be positive were arrayed as in FIG. 2. For each pool, new microtiter plates with HYT media supplemented with 75 μg/mL ampicillin were constructed from all of the colonies which comprise the. Colonies were grown and DNA prepared as in round two.

The mice received both gene gun (wand) and i.m. inoculations at the dosage indicated above. At six weeks, the mice were boosted with 50 μg DNA/animal, but only by i.m. injections. The challenge schedule was the same as in Round Two.

Round Four: *E. coli* from wells at either full by full protection or full by partial protection was streaked out onto YT-plates supplemented with 75 μg/mL ampicillin. Six colonies from each of the plates were tested by PCR colony screening, using the primers FS-UB 5': CCGCACCCTCTCTGATTAC (SEQ ID No: 4) CTGGAGTGGCAACTTCC. (SEQ ID NO. 5) Colonies with different sizes, hence different inserts, were sequenced using ABI Big Dye terminator and the FS-UB primer. Samples were purified on G-50 spin columns, and run on an ABI 377 Sequencer. The generated sequences were analyzed for open reading frames using a program designed by Simon Raynor, Ph.D.

Example 3

Vaccination and Challenge

It was established that the weight increase of the infected lung over the lung weight of naïve, uninfected controls (~ 120 mg) correlated strongly with disease intensity. Maximum disease in this model resulted in approximately 250% lung weight increase, while further lung weight increases were lethal. The lung disease on day 12 after inoculation was characterized by areas of gross lung tissue consolidation and the presence of mononuclear interstitial infiltrates in consolidated tissue. Chlamydial inclusions were observed by immunohistochemistry in many macrophages, but rarely in other cells. Controls for complete protection were established by low level intranasal infection of naïve mice with 3×10^4 IFU of *Chlamydia psittaci* strain B577 4 weeks prior to challenge. These mice were completely protected from disease after challenge infection and had lung weight increases of 10-30% compared to naïve animals. Lungs of completely protected mice did not show gross lung lesions, and pathohistological examination revealed no interstitial infiltrates, but prominent peribronchiolar lymphocytic cuffs, interpreted as sign of protective immune stimulation. The chlamydial lung burden on day 11 after challenge was typically $1\text{-}3 \times 10^6$ IFU per 100mg lung tissue in protected, and $2\text{-}6 \times 10^6$ IFU per 100 mg lung in diseased animals. Since the lowest chlamydial burden was, however, not consistently associated with lowest disease, the inventors used the disease-dependent parameter lung weight rather than chlamydial burden as readout for evaluation of protection. The lung weights were transformed to relative protection scores in a linear equation that assumed the high average lung weight

of the severely ill, naïve, challenged mice as 0 and that of fully protected controls as 1 (FIG. 4).

Example 4

Deconvolution of the Libraries

Since the lung weight was highly variable in the outbred NIH-Swiss mice with variable MHC background, the inventors decided to use inbred BALB/c mice in subsequent rounds. The 48 DNA pools for round two were i.m. injected into BALB/c mice at 50 μg DNA/animal, and the animals were boosted at seven weeks by both gene gun inoculation and i.m. injection. The mice were given a higher *Chlamydia psittaci* challenge, 1.6 x 10^6 IFU *Chlamydia psittaci* B577, at approximately 12 weeks, again to further differentiate the groups. Animals were sacrificed and results evaluated as in round one.

The results of the Round two challenge are depicted in FIG. 4. Of the 48 groups from round two, 15 were judged to be positive, giving a total of 3936 wells. These wells were again arrayed as in round two, but the array had 112 colonies per column and 156 per row with 4-5 colonies per intersection (See FIG. 3). The mice received both gene gun and i.m. injections at the dosage indicated above. At six weeks, the mice were boosted. Both the challenge and the sacrifice were performed as in Round two.

The positive 46 colonies from the intersection wells from Round three were sequenced, and those clones with open reading frames greater than 50 amino acids long were prepared individually and shot into mice as single genes and as a pool. Fourteen clones met these criteria. The disease scoring on each pool in rounds 1-3 are depicted in FIG. 4.

In the fourth round, the animals received two boosts rather than one, and the challenge inoculum was increased to 3 x10^6 IFU *Chlamydia psittaci* B577to increase the selectivity of protection scoring. Furthermore, because too much DNA may lead to a decrease in cellular immune response, the amount of each individual clone was reduced by half but made up the difference with pUC118 DNA, and each mouse received a total 50 μg DNA for i.m. immunization, but only 25 μg of the specific clone. The inventors also decreased the gene gun DNA in the same manner: 1.25 μg/ear of the specific clone

and 1.25 μg pUC118. Mice were boosted i.m. at both four and nine weeks after prime inoculation, and were challenged. The results of this final round are depicted in FIG. 5.

Example 5

Comparison of Clones

Based on the hypothesis that sequences from genes conferring a high level of protection might be selected more than once in the ELI process, the clones were compared against each other for overlaps. Interestingly, one of the clones, CP4 #10, did overlap with another gene, CP4 #11. The gene from which these two clones arise had been partially sequenced (Longbottom et al., 1998).

Two of the genes, CP4 #5 and CP4 #9, had an overlapping region, but they were fused to ubiqutin in opposite orientations. CP4 #5, is composed of two different *Chlamydia psittaci* DNA fragments, fused in opposite orientations. The first gene is fused to ubiqutin in the correct orientation and the correct reading frame. Interestingly, the second gene, which is in the opposite orientation to the ubiqutin gene, has an overlapping sequence to CP4 #5. It is doubtful that the protein from the second gene is produced in the mouse.

Example 6

Analysis of Sequences

The clones conferring protection were re-sequenced and then compared by BLAST search to Genbank and particularly to the recently completed *Chlamydia pneumoniae* (Kalman et al., 1999) genome sequences (FIG. 6). The full-length *Chlamydia psittaci* genes were next isolated and sequences. Upon analysis, all nucleic acid sequences, except #4, #10, #11, and #12, were previously undisclosed in any context. Further, only protions of the sequences encoding #10 and #11 were previously disclosed.

Since most protective genes would not have been predicted by any bioinformatics or information-based approach, it is likely that one will need to apply an unbiased, global approach, such as ELI to define vaccine candidates.

Table 2, lists a comparision of the *Chlamydia psittaci* genes with homologues from *Chlamydia trachomatis* and *Chlamydia pneumoniae*.

Table 2

	Chlamydia ps	*Chlamydia trachomatis*	Identity/ Similarity	*Chlamydia pneumoniae*	Identity/ Similarity
CP4 #1		DNA Pol III Gamma and Tau	62/73	DNA Pol III Gamma and Tau	66/76
CP4 #2		Glu-tRNA Gln Amido-transferase (C subunit)	49/70	Glu-tRNA Gln Amido-transferase (C subunit)	48/63
CP4 #3		Glu-tRNA Gln Amido-transferase (A subunit)	71/85	Glu-tRNA Gln Amido-transferase (A subunit)	71/84
CP4 #4	OMP 90A	Outer Membrane Protein 5	30/45	Outer Membrane Protein G Family Outer Membrane Protein G/I Family	40/54 28/46
CP4 #5		Transglycolase/transpeptidase	67/80	Transglycolase/transpeptidase	67/77
CP4 #6		Protein Translocase	80/89	Protein Translocase	84/92
CP4 #7				Outer Membrane Lipoprotein	60/79
CP4 #8		Oligopeptidase	60/75	Oligopeptidase	61/74
CP4 #9		Hypothetical protein	62/76	Hypothetical protein	62/77
CP4 #10		Outer Membrane Protein 4	27/42	Outer Membrane Protein G family	33/51
CP4 #11		Outer Membrane Protein 4	27/42	Outer Membrane Protein G family	33/51
CP4 #12	OMP 98 kDa	Outer Membrane Protein 5	30/43	Outer membrane Protein G family	44/58
CP4 #13		Protein Translocase	80/89	Protein Translocase	84/92
CP4 #14		Succinate Dehydrogenase	60/76	Succinate Dehydrogenase	61/77

Table 3 lists all of the cloned fragments, their corresponsing full length nucleotide sequences, and the amino acid sequences encoded by both the fragments and the full length sequences. Table 2 further describes the fragments.

TABLE 3—SEQUENCE LISTING INDEX

SEQ ID NO	CP4_NO	Description
SEQ ID NO:6	CP4 #1	(fragment) homolog to *Chlamydia pneumoniae* DNA Pol III Gamma and Tau subunits (dnaX2 gene)
SEQ ID NO:7	CP4 #1	Polypeptide translation corresponding to SEQ ID NO. 6, homolog to *Chlamydia pneumoniae* DNA Pol III Gamma and Tau subunits (dnaX2 gene)
SEQ ID NO:8	CP4 #1	(full length) homolog to *Chlamydia pneumoniae* DNA Pol III Gamma and Tau subunits (dnaX2 gene)
SEQ ID NO:9	CP4 #1	Polypeptide translation corresponding to SEQ ID NO. 8, homolog to *Chlamydia pneumoniae* DNA Pol III Gamma and Tau subunits (dnaX2 gene)
SEQ ID NO:10	CP4 #2	(fragment) homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (C subunit) (gatC gene)
SEQ ID NO:11	CP4 #2	Polypeptide translation corresponding to SEQ ID NO. 10, homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (C subunit) (gatC gene)
SEQ ID NO:12	CP4 #2	(full length) homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (C subunit) (gatC gene)
SEQ ID NO:13	CP4 #2	Polypeptide translation corresponding to SEQ ID NO. 12, homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (C subunit) (gatC gene)
SEQ ID NO:14	CP4 #3	(fragment) homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (A subunit) (gatA gene)
SEQ ID NO:15	CP4 #3	Polypeptide translation corresponding to SEQ ID NO. 14, homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (A subunit) (gatA gene)
SEQ ID NO:16	CP4 #3	(full length) homolog to *Chlamydia pneumoniae* Glu-

		tRNA Gln Amido-transferase (A subunit) (gatA gene)
SEQ ID NO:17	CP4 #3	Polypeptide translation corresponding to SEQ ID NO. 16, homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (A subunit) (gatA gene)
SEQ ID NO:18	CP4 #3	(full length) homolog to *Chlamydia pneumoniae* Glu-tRNA Gln Amido-transferase (B subunit) (gatB gene)
SEQ ID NO:19	CP4 #3	Polypeptide translation corresponding to SEQ ID NO. 18, homolog to *Chlamydia pneumoniae* Glu-Trna Gln Amido-transferase (B subunit) (gatB gene)
SEQ ID NO:20	CP4 #4	(fragment) *Chlamydia psittaci* 90 kDa outer membrane protein (OMP90A gene) (Previously sequenced by Longbottom, et al); homolog to *Chlamydia pneumoniae* Outer Membrane Protein G/I (pmp 9) and Outer Membrane Protein G (pmp 5)
SEQ ID NO:21	CP4 #4	Polypeptide translation corresponding to SEQ ID NO. 20, *Chlamydia psittaci* 90 kDa outer membrane protein (OMP90A gene); homolog to *Chlamydia pneumoniae* Outer Membrane Protein G/I (pmp 9) and Outer Membrane Protein G (pmp 5)
SEQ ID NO:22	CP4 #4	(full length) *Chlamydia psittaci* 90 kDa outer membrane protein (OMP90A gene) (Previously sequenced by Longbottom, et al); homolog to *Chlamydia pneumoniae* Outer Membrane Protein G/I (pmp 9) and Outer Membrane Protein G (pmp 5)
SEQ ID NO:23	CP4 #4	Polypeptide translation corresponding to SEQ ID NO. 22, *Chlamydia psittaci* 90 kDa outer membrane protein (OMP90A gene); homolog to *Chlamydia pneumoniae* Outer Membrane Protein G/I (pmp 9) and Outer Membrane Protein G (pmp 5)
SEQ ID NO:24	CP4 #5	(fragment) homolog to *Chlamydia pneumoniae* transglycolase/transpeptidase (pbp3 gene)
SEQ ID NO:25	CP4 #5	Polypeptide translation corresponding to SEQ ID NO. 24, homolog to *Chlamydia pneumoniae* transglycolase/transpeptidase(pbp3 gene)
SEQ ID NO:26	CP4 #5	(full length) homolog to *Chlamydia pneumoniae* transglycolase/transpeptidase (pbp3 gene)

SEQ ID NO:27	CP4 #5	Polypeptide translation corresponding to SEQ ID NO. 26, homolog to *Chlamydia pneumoniae* transglycolase/transpeptidase (pbp3 gene)
SEQ ID NO:28	CP4 #6	(fragment) homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:29	CP4 #6	Polypeptide translation corresponding to SEQ ID NO. 28, homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:30	CP4 #13	(fragment) homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:31	CP4 #13	Polypeptide translation corresponding to SEQ ID NO. 30, homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:32	CP4 #6 & 13	(full length) homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:33	CP4 #6 & 13	Polypeptide translation corresponding to SEQ ID NO. 32, homolog to *Chlamydia pneumoniae* Protein Translocase (secA2 gene)
SEQ ID NO:34	CP4 #7	(fragment) homolog to *Chlamydia pneumoniae* Outer Membrane Lipoprotein (Cpn 0278)
SEQ ID NO:35	CP4 #7	Polypeptide translation corresponding to SEQ ID NO. 34, homolog to *Chlamydia pneumoniae* Outer Membrane Lipoprotein (Cpn 0278 gene)
SEQ ID NO:36	CP4 #7	(full length) homolog to *Chlamydia pneumoniae* Outer Membrane Lipoprotein (Cpn 0278)
SEQ ID NO:37	CP4 #7	Polypeptide translation corresponding to SEQ ID NO. 36, homolog to *Chlamydia pneumoniae* Outer Membrane Lipoprotein (Cpn 0278 gene)
SEQ ID NO:38	CP4 #8	(fragment) homolog to *Chlamydia pneumoniae* Oligopeptidase (pepF gene)
SEQ ID NO:39	CP4 #8	Polypeptide translation corresponding to SEQ ID NO. 38, homolog to *Chlamydia pneumoniae* Oligopeptidase (pepF gene)

SEQ ID NO:40	CP4 #8	(full length) homolog to *Chlamydia pneumoniae* Oligopeptidase (pepF gene)
SEQ ID NO:41	CP4 #8	Polypeptide translation corresponding to SEQ ID NO. 40, homolog to *Chlamydia pneumoniae* Oligopeptidase (pepF gene)
SEQ ID NO:42	CP4 #9	(fragment) homolog to *Chlamydia pneumoniae* gene of unknown function, co-translationaly coupled to Yop N Flagellar-Type ATPase (Cpn 0708 gene)
SEQ ID NO:43	CP4 #9	Polypeptide translation corresponding to SEQ ID NO. 42, homolog to *Chlamydia pneumoniae* gene of unknown function, co-translationally coupled to Yop N Flagellar-Type ATPase (Cpn 0708 gene)
SEQ ID NO:44	CP4 #9	(full length) homolog to *Chlamydia pneumoniae* gene of unknown function, co-translationally coupled to Yop N Flagellar-Type ATPase (Cpn 0708 gene)
SEQ ID NO:45	CP4 #9	Polypeptide translation corresponding to SEQ ID NO. 44, homolog to *Chlamydia pneumoniae* gene of unknown function, co-translationally coupled to Yop N Flagellar-Type ATPase (Cpn 0708 gene)
SEQ ID NO:46	CP4 #9	(full length) homolog to *Chlamydia pneumoniae* Yop N Flagellar-Type ATPase (yscN gene)
SEQ ID NO:47	CP4 #9	Polypeptide translation corresponding to SEQ ID NO. 46, homolog to *Chlamydia pneumoniae* Yop N Flagellar-Type ATPase (yscN gene)
SEQ ID NO:48	CP4 #10	(fragment) homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene) (Nucleotides 1-423 were previously sequenced by Longbottom *et al.*)
SEQ ID NO:49	CP4 #10	Polypeptide translation corresponding to SEQ ID NO. 48, homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene)
SEQ ID NO:50	CP4 #11	(fragment) homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene) (Nucleotides 1-301 were previously sequenced by Longbottom *et al.*)
SEQ ID NO:51	CP4 #11	Polypeptide translation corresponding to SEQ ID NO. 50,

		homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene)
SEQ ID NO:52	CP4 #10 & 11	(full length) homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene). This gene immediately follows the OMP90A gene on *Chlamydia psittaci*, and nucleotides 1-502 were published by Longbottom *et al.*, although they did not report this as a gene.
SEQ ID NO:53	CP4 #10 & 11	Polypeptide translation corresponding to SEQ ID NO. 52, homolog to *Chlamydia pneumoniae* outer membrane protein G (pmp 2 gene).
SEQ ID NO:54	CP4 #12	(fragment) *Chlamydia psittaci* 98 kDa outer membrane protein (POMP gene) (Previously sequenced by Longbottom, et al)
SEQ ID NO:55	CP4 #12	Polypeptide translation corresponding to SEQ ID NO. 54, *Chlamydia psittaci* 98 kDa outer membrane protein (POMP gene)
SEQ ID NO:56	CP4 #12	(full length) *Chlamydia psittaci* 98 kDa outer membrane protein (POMP gene) (Previously sequenced by Longbottom *et al.*)
SEQ ID NO:57	CP4 #12	Polypeptide translation corresponding to SEQ ID NO. 56, *Chlamydia psittaci* 98 kDa outer membrane protein (POMP gene)
SEQ ID NO:58	CP4 #14	(fragment) homolog to *Chlamydia pneumoniae* Succinate Dehydrogenase (sdhC)
SEQ ID NO:59	CP4 #14	Polypeptide translation corresponding to SEQ ID NO. 58, homolog to *Chlamydia pneumoniae* Succinate Dehydrogenase (sdhC gene)
SEQ ID NO:60	CP4 #14	(full length) homolog to *Chlamydia pneumoniae* Succinate Dehydrogenase (sdhC)
SEQ ID NO:61	CP4 #14	Polypeptide translation corresponding to SEQ ID NO. 60, homolog to *Chlamydia pneumoniae* Succinate Dehydrogenase (sdhC gene)

Of the 14 single genes identified in this study, ten are internal fragments and three contain the C-terminus of the protein. Of the five most protective clones (CP4 #1-5), one was from a putative outer membrane protein (CP4 #4) and one was from a cell surface protein (CP4 #5). The other three were from cytosolic proteins, with CP4 #2 and CP4 #3 deriving independently from genes encoding a particular amidotransferase complex.

Four of the 14 clones have sequence similarity to a class of proteins known as putative outer membrane proteins (POMPs) in *Chlamydia psittaci* and *Chlamydia pneumoniae* (CP4 #4, CP4 #10, CP4 #11 and CP4 #12). Many of the "putative" outer membrane proteins are known to be localized to the outer membrane and to be highly immunogenic (Longbottom et al., 1996; Tan et al., 1990). The clone designated CP4 #4 is an in-frame fragment of POMP90A (Longbottom et al., 1998) and CP4 #12 is an in-frame fragment of a 98 kDa POMP which has been completely sequenced (Accession U72499). The clones CP4 #10 and CP4 #11 immediately follow CP4 #4 in the genome and have sequence similarity to POMPs in *Chlamydia psittaci, Chlamydia trachomatis* and *Chlamydia pneumoniae*. As stated earlier, the clone CP4 #10 overlaps the CP4 #11 clone. Of these clones only CP4 #4 confers significant protection in isolation so clearly the criteria of being an outer membrane protein is not sufficient to predict a protective vaccine.

Example 7

Mixing Experiment

The two dimensional approach used to find protective gene fragments assumes that the protection is due to a single highly protective gene within a pool. To verify that such genes would be found, 25 ng (i.e. 1/2000) of either CP4 #4 or CP4 #11 was added to a pool that scored negative (pool 6 round 1). As depicted in FIG. 7, spiking with either clone converted the negative library to a positive. Of note is that CP4 #11 did not confer protection when tested individually, however, it does protect in combination.

The fact that a CP4 #4 positive library confers protection validates the sensitivity of the system. The fact that a CP4 #11 positive library protects implies that CP4 #11 con be a useful component of a vaccine, but that it may depend upon having other antigens

present. A likely explaination is that CP4 #11 is a good vaccine antigen, but requires immunological help.

Example 8

Vaccination in Cattle

An important question is whether the genes identified in this manner in a mouse model are clinically relevant. Of course, this concern is not peculiar to genetic vaccines or ELI, but any system that uses models to identify vaccine candidates. In this case the clinically relevant situation is protection of cattle. In a preliminary experiment, the inventors evaluated the pool of 14 individual clones in the original host in a fertility challenge model. All fourteen clones were used as the individual test data on each clone in mice was not available by the time it was necessary to initiate the cow trial.

TABLE 4

***Chlamydia psittaci* Vaccine in Cows**

	Percent Pregnant	Pregnant	Not Pregnant
Not Challenged	75	3	1
Challenged, Not Vaccinated	0	0	4
EB Vaccine	25	1	3
Genetic Vaccine (14 gene pool)	33	2	4

Chlamydia psittaci is normally introduced by the fecal-oral and respiratory routes in cattle, and disseminates to other tissues including reproductive organs. *Chlamydia psittaci* infection of the uterine mucosa reduces fertility, the basis of the economic interest in a *Chlamydia psittaci* vaccine. Four groups of heifers were used. One group was the naïve unchallenged control, another the naïve, challenged control, a third received the same pool of fourteen gene fragments that were tested in mice, and the fourth group was vaccinated with an experimental, inactivated vaccine of elementary bodies (EB) and also challenged. This EB vaccine had shown great promise in field trials but is too expensive to produce. After a prime and one boost, the heifers were estrus synchronized by prostaglandin injection, were in heat 2-3 days later, and were artificially inseminated, simultaneously receiving an intracervical chlamydial challenge of $3x10^7$ inclusion forming units. The heifers were palpated for pregnancy at six weeks after insemination. This challenge was very high in order to maximize the difference between positive and negative control animals. This was necessary because only a small number of cows could be justified for this high-risk experiment.

Although the animal numbers are small, the results are quite encouraging. As is seen in Table 4, three out of four animals became pregnant in the positive control (non-challenged) group, 0/4 in the negative control (non-vaccinated, challenged) group, 2/6 in the genetic immunization group, and 1/4 in the elementary body vaccine group. The genetic vaccine of the pooled genes performed at least as well as the EB vaccine. Also relative to the inventor's interest in therapeutic vaccines, these cows were not sterile with respect to *Chlamydia psittaci* at the time of the prime inoculation. The vaccination was in the face of previous exposure and low level *Chlamydia psittaci* infection, as determined by the high titers of preinoculation antichlamydial antibodies, and occasional positivity of *Chlamydia ompl* PCRs from vaginal scrapings.

The next phase in developing a cow vaccine will be to experimentally verify the effectiveness of particular groups of the protective genes and then convert the codon usage of the *Chlamydia psittaci* genes to that of a mammal. This should increase the expression of the antigen in cows and increase the effectiveness of the vaccine. The inventors will test different combinations of those genes which have been found to be individually protective, as well as combinations with CP4 #11. Both the original fragments and their full-length versions can be tested, both as nucleic acid segments and proteins. Once the combinations have been verified in mice or other small mammals, those combinations showing the most promise will be tested in cows. After immunization, the cows will be challenged with *Chlamydia psittaci*, either by direct challenge at insemination or infection by herd-mates. Direct challenge at insemination is a very severe and unnatural form of challenge. Therefore, even if protection is not demonstrated in the wake of such challenge, this does not necessarily mean that no protection has been conferred upon the cows.

Example 9

Fertility at 42 days post breeding in heifers vaccinated with the pool of the 5 best mouse-protective genes of *Chlamydia psittaci.*

Because it is known that bacterial genes are not expressed efficiently in mammalian cells, the five most protective genes were chemically resynthesized to give an optimal mammalian codon bias. In addition, the full-length genes corresponding to the fragments isolated in the screen were used.

One group of five heifers was vaccinated with this pool. Another group of six heifers was vaccinated with an Alum-Quil A based vaccine containing per dose 100 μg each of the affinity-purified protein fragments expressed in *E. coli* from these genes. The control group of twelve heifers was vaccinated with a plasmid expressing an unrelated bacterial gene. Six weeks after the initial immunization all groups received booster vaccinations. Eight weeks later all heifers, including a cohort of 27 non-vaccinated heifers, were estrus-synchronized by prostaglandin injection. After coming into heat two to three days later, the non-vaccinated cohort heifers were infected with an intrauterine

chlamydial inoculum of 10^8 IFUs *C. psittaci* B577. The function of this group was to shed chlamydiae, and thus to challenge through natural infection routes the vaccinated animals at the time of breeding. Eleven days later, the vaccinated animals were re-synchronized, and inseminated at estrus. The heifers were rectally palpated for pregnancy determination at six weeks after insemination.

The Genetic Vaccine group was vaccinated with DNA comprised of the pool of 5 full length, mammalianized genes, the Protein Vaccine group with the 5 full-length proteins, and the control group with DNA of an unrelated gene from *Salmonella typhimurium*. During the 3-week period prior to *C. psittaci* infection, heifers of all groups, including the non-vaccinated challenge cohort, shed low levels of *C. psittaci* (0.5±0.2 genomes/swab) as determined by qPCR of weekly collected vaginal cytobrush swabs. To challenge the vaccinated animals via natural transmission at the time of breeding, a cohort of 27 non-vaccinated animals was intracervically infected with *C. psittaci*. Eleven days later, all vaccinated groups were estrus-synchronized and inseminated. During the 4 weeks following the infection, the infected cohort animals shed high levels of chlamydiae (3826±2052 genomes per swab), and then returned to low baseline shedding (24.2±10.9 genomes per swab) for the remaining 5-week observation period. All vaccinated heifers were exposed to the natural challenge infection, as evident in their 7-fold increased post-breeding shedding of chlamydiae (3.6±1.2 genomes/swab; $p < 0.05$) compared to pre-breeding shedding of all heifers. No difference in chlamydial shedding before or after breeding was found between the *C. psittaci* vaccinated and the control vaccinated groups.

TABLE 5. Fertility in cows vaccinated with a pool of the 5 best mouse-protective *Chlamydia psittaci* genes.

Group	Percent Pregnant	Pregnant	Not Pregnant
Control Group	50	6	6
Genetic Vaccine	80	4	1
Protein Vaccine	83	5	1

As is seen in Table 5, six out of twelve animals (50 % fertility) became pregnant in the control group, 4/5 or (80 % fertility in the genetic vaccine group, and five out of six

(83% fertility) in the protein vaccine group. Thus, 9/11 animals in both vaccine groups were pregnant. The genetic vaccine of the pooled genes performed as well as the protein vaccine.These fertility data correspond very well with typical data of bovine herds with and without fertility problems. When both vaccine groups combined are compared to the controls, the 1-tailed Fisher's exact test indicates with a p = 0.122 that vaccination is effective to improve *Chlamydia*-induced reduction of fertility. The odds ratio for improvement of fertility by vaccination is 4.5 (0.67-30.23, 95% confidence interval). These data are important in view of the fact that all heifers in the experiment had been previously exposed to chlamydiae and experienced low-level herd infection with *C. psittaci,* as determined by positive *C. psittaci* B577 MOMP-peptide ELISA and sporadic detection by quantitative PCR of low levels of *C. psittaci* in pre-challenge vaginal cytobrush swabs.

Example 10

Creation and Testing of Vaccines Using *Chlamydia psittaci* Nucleic Acid and Amino Acid Sequences To Protect Non-Bovine Species

The *Chlamydia psittaci* sequences and antigens disclosed in this application are envisioned to be used in vaccines for *Chlamydia psittaci* in commercially important animals such as dairy cattle. Field trials in cattle are being conducted, as described above. However, these *Chlamydia psittaci* sequences may be used to create vaccines for other species as well, including other species of *Chlamydia* and other bacterial pathogens.

For example, one may use the information gained concerning *Chlamydia psittaci* to identify a sequence in another bacterial pathogen that had substantial homology to the *Chlamydia psittaci* sequences. In many cases, this homology would be expected to be more than 30% amino acid sequence identity or similarity and could be for only part of a protein, e.gg 30 amino acids, in the other species. The gene encoding such identity/similarity may be isolated and tested as a vaccine candidate in the appropriate model system either as a protein or nucleic acid. Alternatively, the *Chlamydia psittaci* homologs may be tested directly in an animal species of interest since having so few genes to screen (10 or less) and given that the genes had been demonstrated to be protective in another species the probability of success would be high. Alternatively,

proteins or peptides corresponding to the homologs to the *Chlamydia psittaci* genes may be used to assay in animals or humans for immune responses in people or animals infected with the relevant pathogen. If such immune responses are detected, particularly if they correlated with protection, then the genes, proteins or peptides corresponding to the homologs may be tested directly in animals or humans as vaccines.

Example 11

Creation and Testing of Commercial Vaccines Using *Chlamydia psittaci* Nucleic Acid and Amino Acid Sequences

The genes identified and claimed as vaccine candidates can be developed into commercial vaccines in the following manner. The genes identified can be converted to optimized mammalian expression sequences by changing the codons. This is a straightforward procedure, which can be easily do by one of skill in the art, and has been done for the *Chlamydia psittaci* sequences. The genes can then be tested in the relevant host, for example, cattle, for the relevant protection, for example, fertility. Genetic immunization affords a simple method to test vaccine candidate for efficacy and this form of delivery has been used in a wide variety of animals including humans. Alternatively, the genes may be transferred to another vector, for example, a vaccinia vector, to be tested in the relevant host in this form. Alternatively, the corresponding protein, with or without adjuvants may be tested. These tests may be done on a relatively small number of animals. Once conducted, a decision can be made as to how many of the protective antigens to include in a larger test. Only a subset may be chosen based on the economics of production. A large field trial may be conducted using the formulation arrived at. Based on the results of the field trial, possibly done more than once at different locations, a commercial vaccine would go into production.

Example 12

Creation and Testing of Vaccines Against Other Pathogens Using *Chlamydia* Nucleic Acid and Amino Acid Sequences

Since *Chlamydia pneumonia* has a similar pathobiology as *Chlamydia psittaci*, the inventors take advantage of the screening already accomplished on the *Chlamydia*

psittaci genome to test *Chlamydia pneumoniae* for homologs corresponding to the ones from *Chlamydia psittaci* as vaccine candidates. Those of ordinary skill may expect that, as one moved evolutionarily away from *Chlamydia psittaci*, the likelihood that the homologs would protect would presumably decline. However, researchers would be likely to test the homologs identified from even disparate species for protective ability in regard to relevant diseases, as this could reduce the search of a genome for vaccine candidates ~200-1,000 fold. Once the homologs have been identified and isolated, they may be tested in the appropriate animal model system for efficacy as a vaccine. For example, the *Chlamydia pneumonia* homologs as genes or proteins can be tested in a mouse pneumonia model or in a mouse or rabbit atherosclerosis model.

In an example, showing the applicability of the use of homology to determine protective antigens in differing genera, it has been shown that hsp65, the *Mycobacterium tuberculosis* homolog of the *Chlamydia pneumonia* hsp60 gene, is protective against *Mycobacterium tuberculosis*, just as hsp60 is protective against *Chlamydia pneumonia*. This validates that homologous genes from two different pathogens can result in protective genetic vaccines against those pathogens. Therefore, there is a strong impetus to use the *Chlamydia* gene sequences that have been disclosed as protective herein, and other such sequences that may be determined by the methods disclosed herein, to search for protective sequences of other species.

To prove this concept, full length gene of *Chlamydia pneumonia* homolog of *Chlamydia psittacii* underwent PCR and the animals were challenged with Chlamydia pneumonia. The gene that conferred protection against *Chlamydia psittaci* gave the best protection against *Chlamydia pneumonia*. As demonstrated in FIG. 8 and Table 6, the genes of *Chlamydia pneumonia dnaX2* (SEQ. ID NO 62), gatA (SEQ. ID NO 64); pbp3 (SEQ. ID NO 66); and the unknown gene 0278 (SEQ. ID NO 68), and their respective amino acid sequences (SEQ. ID NO 63, SEQ. ID NO 65, SEQ. ID NO 67, and SEQ. ID NO 69), conferred protection against *Chlamydia pneumonia*.

Table 6. Protection (log of colonies in lung) against *Chlamydia pneumoniae*

Name of gene	Protection
Vaccinated Ctr	5.3
**C.pn. dnax2	5.4
C.pn. gatC	7.3
**C.pn. gatA	6.5
C.pn. Pmp5	7.4
C.pn. Pmp9	7.6
**C.pn. Pbp3	6.4
C.pn. SecA	7.2
**C.pn. Unk.0278	6.7
C.pn. pepF	7.2
C.pn. 0708	7.2
C.pn. Pmp2	7.0
pool, 5 best C.psittaci	7.0
Irrelev. Vacc.	6.9

** genes conferred protection

The above study indicates that, once one of ordinary skill has access to the *Chlamydia* sequences disclosed in this specification, or to additional sequences determined to be protective using any of the methods disclosed in this specification, it is easy to run a computer-based search of relevant genetic databases in order to determine homologous sequences in other pathogens. For example, these searches can be run in the BLAST database in GenBank.

Once a sequence which is homologous to a protective sequence is determined, it is possible to obtain the homologous sequence using any of a number of methods known to those of skill. For example, it is easy to PCR amplify the pathogen homolog genes from genomic DNA and clone the genes into an appropriate genetic immunization vector, such as those used for ELI. These homolog genes can then be tested in an animal model appropriate for the pathogen for which protection is sought, to determine whether homologs of the *Chlamydia* genes will protect a host from challenge with that pathogen.

For example, the dnaX2 gene from *Chlamydia psittaci* is homologous to the dnaX2 gene from *Helicobacter pylori*. Therefore, one can will amplify the dnaX2 gene from *Helicobacter pylor* genomic DNA and clone it into a genetic immunization vector. The clone could then be tested for protection by inoculating animals with the *Helicobacter pylor* dnaX2 clone, then challenging the inoculated animals with *Helicobacter pylor* bacteria.

Of course, it is possible for one of ordinary skill to use the *Chlamydia* genes that are disclosed as protective herein, or determined to be protective using the methods disclosed herein, to obtain protective sequences from a first non-*Chlamydia* organism, then to use the protective sequences from the non-*Chlamydia* organism to search for homologous sequences in a second non-*Chlamydia* or *Chlamydia* organism. So long as a protective *Chlamydia* sequence is used as the starting point for determining at least one homology in such a chain of searches and testing, such methods are within the scope of this invention.

* * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U. S. Patent No. 5,670,488

U. S. Patent No. 5,770,414

U.S. Patent No. 5,824,544

U.S. Patent No. 5,830,725

U.S. Patent No. 5,851,826

U.S. Patent No. 5,858,744

U.S. Patent No. 5,879,934

U.S. Patent No. 5,888,502

U.S. Patent No. 5,932,210

U.S. Patent No. 5,955,331

Amado and Chen, "Lentiviral vectors—the promise of gene therapy within reach?" *Science*, 285(5428):674-676, 1999.

Amado, Mitsuyasu, Zack, "Gene therapy for the treatment of AIDS: animal models and human clinical experience," *Front Biosci.*, 4:D468-475, 1999.

Babiuk, L. A. "Broadening the approaches to developing more effective vaccines," *Vaccine 17*, 1587-95, 1999.

Bangham *et al.*, *J. Mol. Biol.*, 13:238, 1965.

Barry, M. A., Lai, W. C., and Johnston, S. A. Protection against mycoplasma infection using expression-library immunization. Nature *377*, 632-5, 1995.

Batra, Guttridge, Brenner, Dubinett, Baldwin, Boucher, "IkappaBalpha gene transfer is cytotoxic to squamous-cell lung cancer cells and sensitizes them to tumor necrosis factor-alpha-mediated cell death," *Am. J. Respir. Cell Mol. Biol.*, 21(2):238-245, 1999.

Bett, Prevec, Graham, "Packaging capacity and stability of human adenovirus type 5 vectors," *J. Virol.*, 67(10):5911-5921, 1993.

Blackwell, Miller, Douglas, Li, Peters, Carroll, Peters, Strong, Curiel, "Retargeting to EGFR enhances adenovirus infection efficiency of squamous cell carcinoma," *Arch. Otolaryngol Head Neck Surg.*, 125(8):856-863, 1999.

Brayton, K. A., Vogel, S. W., and Allsopp, B. A. Expression library immunization to identify protective antigens from Cowdria ruminantium. Ann N Y Acad Sci *849*, 369-71, 1998.

Campbell, L. A., C.-C., K., and Grayston, J. T. *Chlamydia* pneumoniae and cardiovascular disease. Emerging Infectious Disease *4*, 571-579, 1998.

Carrio, Romagosa, Mercade, Mazo, Nadal, Gomez-Foix, Fillat, "Enhanced pancreatic tumor regression by a combination of adenovirus and retrovirus-mediated delivery of the herpes simplex virus thymidine kinase gene," *Gene Ther.*, 6(4):547-553, 1999.

Case, Price, Jordan, Yu, Wang, Bauer, Haas, Xu, Stripecke, Naldini, Kohn, Crooks, "Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors,""*Proc. Natl. Acad. Sci. USA*, 96(6):2988-2993, 1999.

Chillon, Bosch, Zabner, Law, Armentano, Welsh, Davidson, "Group D adenoviruses infect primary central nervous system cells more efficiently than those from group C," *J. Virol.*, 73(3):2537-2540, 1999.

Clay, Custer, Spiess, Nishimura, "Potential use of T cell receptor genes to modify hematopoietic stem cells for the gene therapy fo cancer," *Pathol. Oncol. Res.*, 5(1):3-15, 1999.

Danesh, J., Collins, R., and Peto, R. . Chronic infections and coronary heart disease: is there a link? [see comments]. Lancet *350*, 430-6, 1997.

Deamer and Uster, "Liposome Preparation: Methods and Mechanisms," *LIPOSOMES*, M. Ostro ed. 1983.

Derby, Sena-esteves, Breakefield, Corey, "Gene transfer into the mammalian inner ear using HSV-1 and vaccinia virus vectors," *Hear Res.*, 134(1-2):1-8, 1999.

Dongji, Z., Xi, Y., Caixia, S., and Brunham, R. C. The immune responses following intramuscular DNA immunization with the MOMP gene of *Chlamydia* trachomatis. In Chlamydial Infections: Proceedings of the 9th International Symposium on Human Chlamydial Infection., e. a. R. Stephens, ed. (Napa, California: University of California Press, Berkeley, USA.), pp. 442-445, 1998.

Dorai, Perlman, Walsh, Shabsigh, Goluboff, Olsson, Buttyan, "A recombinant defective adenoviral agent expressing anti-bcl-2 ribozyme promotes apoptosis of bcl-2-expressing human prostate cancer cells," *Int. J. Cancer*, 82(6):846-852, 1999.

Ellis, R. W. (1999). New technologies for making vaccines. Vaccine *17*, 1596-604.

Engel and Kohn, "Stem cell directed gene therapy," *Front Biosci.*, 4:e26-33, 1999.

Feldman, Tahlil, Steg, "Adenovirus-mediated arterial gene therapy for restenosis: problems and perspectives," *Semin Interv. Cardiol.*, 1(3):203-208, 1996.

Feltquate, D. M., Heaney, S., Webster, R. G., and Robinson, H. L. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J Immunol *158*, 2278-84, 1997.

Fujiwara and Tanaka, "Molecular surgery for human colorectal cancer with tumor suppressor p53 gene transfer," *Nippon Geka gakkai Zasshi*, 99(7):463-468, 1998.

Garrido, Carnicero, Lim, Schimmang, "Differential effects on the survival of neuronal and non-neuronal cells after infection by herpes simplex virus type 1 mutants," *J. Neurovirol.*, 5(30)280-288, 1999.

Ghosh and Bachhawat, "Targeting of liposomes to hepatocytes," *In:* Wu G. Wu C ed., Liver diseases, targeted diagnosis and therapy using specific receptors and ligands, New York: Marel Dekker, pp. 87-104, 1991.

Graham and Prevec, "Methods for construction of adenovirus vectors," *Mol. Biotechnol.*, 3(3):207-220, 1995.

Grayston, J. T., Woolridge, R. L., and Wang, S.-P. Trachoma vaccine studies on Taiwan. Ann. NY Acad. Sci. *98*, 352-362, 1962.

Gregoriadis, *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis (ed.), pp. 287-341, 1979.

Haecker, Stedman, Balice-Gordon, Smith, Greelish, Mitchell, Wells, Sweeney, Wilson, "*In vivo* expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes,""*Hum. Gene Ther.*, 7(15):1907-1914, 1996.

Hermens and Vergaagen, "Viral vectors, tools for gene transfer in the nervous system," *Prog. Neurobiol.*, 55(4):399-432, 1998.

Howard, Kalthoff, Fong, "Ablation of tumor cells *in vivo* by direct injection of HSV-thymidine kinase retroviral vector and ganciclovir therapy," *Ann. N.Y. Acad. Sci.*, 880-352-365, 1999.

Huang, J., Wang, M.-D., Lenz, S. D., Gao, D., and Kaltenboeck, B. Interleukin-12 administered during *Chlamydia* psittaci lung infection in mice confers immediate and long-term protection and reduces MIP-2 level and neutrophil infiltration in lung tissue. J. Immunol. *162*, 2217-2226, 1999.

Ilan, Saito, Thummala, Chowdhury, "Adenovirus-mediated gene therapy of liver diseases," *Semin Liver Dis.*, 19(1):49-59, 1999.

Irie, Anderegg, Kashani-Sabet, Ohkawa, Suzuki, Halks-Miller, Curiel, Scanlon, "Therapeutic efficacy of an adenovirus-mediated anti-H-ras ribozyme in experimental bladder cancer," *Antisense Nucleic Acid Drug Dev.*, 9(4):341-349, 1999.

Johnston, Gasmi, Lim, Elder, Yee, Jolly, Campbell, Davidson, Sauter, "Minimum requirements for efficient trandsduction of dividing and nondividing cells by feline immunodeficiency virus vectors," *J. Virol.*, 73(6):4991-5000, 1999.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R. W., Olinger, L., Grimwood, J., Davis, R. W., and Stephens, R. S. Comparative genomes of *Chlamydia* pneumoniae and C. trachomatis. Nature Genetics *21*, 385-389, 1999.

Kaltenbock, B., Schmeer, N., and Schneider, R. Evidence for numerous ompl alleles of porcine *Chlamydia* trachomatis and novel chlamydial species obtained by PCR. J Clin Microbiol *35*, 1835-41, 1997.

Kaltenboeck, B., Huang, J., Wang, M.-D., Lenz, S. D., and Gao, D. Genetically determined vigorous innate immunity is associated with protection against primary chlamydial lung infection in mice, but with profound disease exacerbation in reinfection. In Chlamydial Infections: Proceedings of the 9th

International Symposium on Human Chlamydial Infection, e. a. R. Stephens, ed. (Napa, California: University of California Press), 1998.

Kaltenboeck, B., Kousoulas, K. G., and Storz, J. Structures of and allelic diversity and relationships among the major outer membrane protein (ompA) genes of the four chlamydial species. J Bacteriol *175*, 487-502, 1993.

Kaneda *et al.*, "Increased expression of DNA cointroduced with nuclear protein in adult rat liver," *Science*, 243:375-378, 1989.

Kato *et al.*, "Expression of hepatitis B virus surface antigen in adult rat liver," *J. Biol. Chem.*, 266:3361-3364, 1991.

Kaufman, Jia, Tan, Chen, Gabelt, Booth, Tufaro, Cynader, "A perspective of gene therapy in the glaucomas," *Surv. Ophthalmol.*, 43(1S):91-97, 1999.

Kay, "Hepatic gene therapy for haemophilia B.," *Haemophilia*, 4(4):389-392, 1998.

Klimatcheva, Rosenblatt, Planelles, "Lentiviral vectors and gene therapy," *Front Biosci.*, 4:D481-496, 1999.

Lachmann and Efstathiou, "Use of herpes simplex virus type 1 for transfene expression within the nervous system," *Clin. Sci.*, 96(6):533-541, 1999.

Leibowitz, Beattie, Kafri, Cirulli, Lopez, Hayek, Levine, "Gene transfer to human pancreatic endocrine cells using viral vectors," 48(4):745-753, 1999.

Lesch, "Gene transfer to the brain: emerging therapeutic strategy in psychiatry," *Biol. Psychiatry*, 45(3):247-253, 1999.

Longbottom, D., Russell, M., Dunbar, S. M., Jones, G. E., and Herring, A. J. Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia* psittaci subtype that causes abortion in sheep. Infect Immun *66*, 1317-24, 1998

Longbottom, D., Russell, M., Jones, G. E., Lainson, A., and Herring, A. J. Identification of a multigene familiy coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia* psittaci. FEMS Microbiology Letters *142*, 277-281, 1996.

Marienfeld, Haack, Thalheimer, Schneider-Rasp, Brackmann, Poller, "Autoreplication of the vector genome in recombinant adenorviral vectors with different E1 region deletions and transgenes, *Gene Ther.*, 6(6):1101-1113, 1999.

Miller, Miller, Garcia, Lynch, "Use of retroviral vectors for gene transfer and expression," *Methods Enzymol.*, 217:581-599, 1993.

Miyake, Suzuki, Matsuoka, Tohyama, Shimada, "Stable integration of human immunodeficiency virus-based retroviral vectors into the chromosomes of nondividing cells," *Hum. Gene Ther.*, 9(4):467-475, 1998.

Miyatake, Tani, Feigenbaum, Sundaresan, Toda, Narumi, Kikuchi, Hashimoto, Hangai, Martuza, Rabkin, "Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus *in vivo*,'*Gene Ther.*, 6(4):564,572, 1999.

Moldawer, Edwards, Josephs, Minter, Copeland, MacKay, "Application of gene therapy to acute inflammatory diseases," *Shock*, 12(2):83-101, 1999.

Morrison, Onions, Nicolson, "Complete DNA sequence of canine adenovirus type 1," *J. Gen. Virol.*,78(Pt4):873-878, 1997.

Morrison, R. P., Feilzer, K., and Tumas, D. B. Gene knockout mice establish a primary protective role for major histocmpatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. *Infect. Immun. 63*, 4661-4668, 1995.

Morrison, R. P., J., B. R., Lyng, K., and Caldwell, H. D. *Chlamydia* disease pathogenesis. The 57-kDa chlamydial hypersensitivity antigen is a stress response protein. J Exp Med *170*, 1271, 1989.

Morrison, R. P., Lyng, K., and Caldwell, H. Chlamydial disease pathogenesis: ocular hypersensitivity elicited by a genus-specific 57-kD protein. J. Exp. Med. *169*, 663-675, 1989.

Muhlestein, J. B. Bacterial infections and atherosclerosis. J. Invest. Med. *46*, 396-402, 1998.

Nicolau *et al.*, "Liposomes as carriers for *in vivo* gene transfer and expression," *Methods Enzymol.*, 149:157-176, 1987.

Pal, S., Barnhart, K. M., Wei, Q., Abai, A. M., Peterson, E. M., and de la Maza, L. M. Vaccination of mice with DNA plasmids coding for the *Chlamydia* trachomatis major outer membrane protein elicits an immune response but fails to protect against a genital challenge. Vaccine *17*, 459-65, 1999.

Petrof, "Respiratory muscles as a target for adenovirus-mediated gene therapy," *Eur. Respir. J.*, 11(2):492-497, 1998.

Piedrafita, D., Xu, D., Hunter, D., Harrison, R. A., and Liew, F. Y. Protective immune response induced by vaccination with an expression genomic library of Leishmania major. Journal of Immunology *163*, 1467-1472, 1999.

Quinn Does *Chlamydia* pneumoniae cause coronary heart disease? Current Opinions in Infectious Diseases *11*, 301-307, 1998.

Rabinovitch, Suarez-Pinzon, Strynadka, Ju, Edelstein, Brownlee, Korbutt, Rajotte, "Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction," *Diabetes*, 48(6):1223-1229, 1999.

Reddy, Idamakanti, Zakhartchouk, Baxi, Lee, Pyne, Babiuk, Tikoo, "Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3," *J. Virol.*, 72(2):1394-1402, 1998.

Robbins, Tahara, Ghivizzani, "Viral vectors for gene therapy," *Trends Biotech.*., 16(1):35-40, 1998.

Rottenberg, M. E., Gigliotti Rothfuchs, A. C., Gigliotti, D., Svanholm, C., Bandtholtz, L., and Wigzell, H. Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *J. Immunol.*, 2829-2836, 1999.

Sanford, J. C., Devit, M. J., Russel, J. A., Smith, F. D., Harpending, P. R., Roy, M. K., and Johnston, S. A. An improved, helium-driven biolistic device. Technique *3*, 3-16, 1991.

Schachter, J. Overview of human diseases. In Microbiology of *Chlamydia*, A. L. Barron, ed. (Boca Raton, FL: CRC Press, Inc.), pp. 153-165, 1988.

Smith, "Adenovirus-mediated gene transfer to treat neurologic disease," *Arch. Neurol.*, 55(8):1061-1064, 1998.

Stamm, W. E. *Chlamydia* trachomatis infections: progress and problems. J. Inf. Dis. *179 Suppl 2*, S380-383, 1999.

Stewart, Lassam, Quirt, Bailey, Rotstein, Krajden, Dessureault, Gallinger, Cappe, Wan, Addison, Moen, Gauldie, Graham, "Adenovector-mediated gene delivery of

interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial," *Gene Ther.*, 6(3):350-363, 1999.

Storz, J. Antigenic structures and interrelations of PL agents associated with polyarthritis, enzootic abortion, intrauterine and latent intestinal infections. J. Comp. Pathol. *76*, 351, 1966.

Storz, J., and Kaltenboeck, B. Disease diversity of chlamydial infections. In Rickettsial and chlamydial diseases of domestic animals, Z. Woldehiwet and M. Ristic, eds. (Oxford, UK: Pergamon Press), pp. 363-392, 1993.

Sykes, K. F., and Johnston, S. A. Genetically-live vaccines mimic the antigenicity but not pathogenicity of live viruses. DNA Cell. Biol. *18*, 521-531, 1999.

Szoka and Papahadjopoulos, *Proc. Nat'l Acad. Sci. U.S.A.* 75:4194-98, 1978.

Takahashi, Miyoshi, Verma, Gage, "Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer," *J. Virol.*, 73(9):7812-7816, 1999.

Tan, T. W., Herring, A. J., Anderson, I. E., and Jones, G. E. Protection of sheep against *Chlamydia* psittaci infection with a subcellular vaccine containing the major outer membrane protein. Infect Immun *58*, 3101-8 , 1990.

Tang, D. C., DeVit, M., and Johnston, S. A. Genetic immunization is a simple method for eliciting an immune response. Nature *356*, 152-4, 1992.

Vanderkwaak, Wang, Gomez-Navarro, Rancourt, Dmitriev, Krasnykh, Barnes, Siegal, Alvarez, Curiel, "An advanced generation of adenoviral vectors selectively enhances gene transfer for ovarian cancer gene therapy approaches," *Gynecol. Oncol.*, 74(2):227-234, 1999.

Vanrompay, D., Cox, E., Vandenbussche, F., Volckaert, G., and Goddeeris, B. Protection against *Chlamydia* psittaci challenge by gene gun-based DNA immunizations. Vaccine *17*, 2628-2635, 1999.

Wang, R., Doolan, D. L., Charoenvit, Y., Hedstrom., R. C., Gardner, M. J., Hobart, P., Tine, J., Sedegah, M., Fallarme, V., Sacci jr., J. B., Kaur, M., Klinman, D. M., Hoffman, S. L., and Weiss, W. R. Simultaneous induction of multiple antigen-specific cytoxic T lymphocytes in nonhuman primates by immunization with a

mixture of four Plasmodium falciparum DNA plasmids. Infec. Immun. *66*, 4193-4202, 1998.

Weihl, Macdonald, Stoodley, Luders, Lin, "Gene therapy for cerebrovascular disease," *Neurosurgery*, 44(2):239-252, 1999.

White, Renda, Nam, Klimatcheva, Zhu, Fisk, Halterman, Rimel, Federoff, Pandya, Rosenblatt, Planelles, "Lentivirus vectors using human and simian immunodeficiency virus elements," *J. Virol.*, 73(4):2832-2840, 1999.

Wilson, "Adenoviruses as gene-delivery vehicles," *N. Engl. J. Med.*, 334(18):1185-1187, 1996.

Wong *et al.*, "Appearance of β-lactamase activity in animal cells upon liposome mediated gene transfer," *Gene*, 10:87-94, 1980.

Wu, "Recent advances in gene therapy of GI and liver diseases," *Chung Hua Min Kuo Hsiao Erh Ko I Hsueh Hui Tsa Chih*, 39(5):297-300, 1998.

Yeung, Bockhold, Tufaro, "Efficient infection of mature skeletal muscle with herpes simplex virus vectors by using dextran sulfate as a co-receptor," *Gene Ther.*, 6(9):1536-1544, 1999.

Yoon, Carroll, Chiocca, Tanabe, "Influence of p53 on herpes simplex virus type 1 vectors for cancer gene therapy," *J. Gastrointest. Surg.*, 3(10):34-48, 1999.

Zheng, Graham, Prevec, "Transcription units of E1a, E1b and pIX regions of bovine adenovirus type 3," *J. Gen. Virol.*, 80(Pt7):1735-1742, 1999.